School of Molecular and Life Sciences

‘A Spot of Bother’

Pyrenophora teres f. *maculata:* Intercontinental population structure, genetic diversity, and selection influencing regional populations

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Declaration

To the best of my knowledge and belief, this thesis contains no material previously published by any other person except where due acknowledgement has been made. This thesis contains published works and work prepared for publication. The thesis contains no material that has been accepted for the award of any other degree or diploma in any university.

The research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007) – updated March 2014. The proposed research study received human research ethics approval from the Curtin University Human Research Ethics Committee (EC00262), Approval Number #HRE2020-0459.

Student signature:

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Date:

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Abstract

Pyrenophora teres, the fungal pathogen responsible for net blotch in barley, significantly impacts the Australian barley industry with annual losses exceeding $300 million (Murray & Brennan, 2010). It manifests as two forms, *P. teres* f. *teres* (*Ptt*) causing net form net blotch (NFNB) and *P. teres* f. *maculata* (*Ptm*) causing spot form net blotch (SFNB). This thesis aims to address knowledge gaps by examining genetic relationships between *Ptm* populations, both nationally and internationally. Further, the genetic status of *P. teres* of barley grass was also addressed using modern molecular analysis techniques.

Initially I conducted a comprehensive genetic characterisation of the most geographically diverse *Ptm* population examined within Australia to date. I employed a genome-wide marker system, DArTseq, using 1252 SNP markers to characterise 254 Australian isolates. Analysis revealed genotypic diversity and an absence of population structure, either between states, or between fields and cultivars in different agro-ecological zones. However, two cryptic genotypic groups were found only in Western Australia. These groups were associated with genes involved in fungicide resistance suggesting an alternative untested mechanism for population structure in Australia.

In the next study, the analysis was expanded to characterise the intercontinental population structure of *Ptm,* including representative isolates from Australia, the Republic of South Africa (RSA), Hungary, Turkey, Canada, and a single historical isolate from Denmark. It was found that there was generally a defined population structure between countries with a high degree genetic similarity shared between Western Australian and South African populations. The genetic similarities coincided with the population substructure resolved in the first stage, revealing that a small subgroup of WA isolates was likely related to the RSA population sharing genomic regions associated to fungicide resistance.

In the final study, *P. teres* isolated from barley grass (*Hordeum leporinum*) growing alongside barley (*Hordeum vulgare*) were analysed, aiming to characterise their genetic and symptomatic relationship to *P. teres* isolates from barley. The genetic diversity differences between *P. teres* collected from barley grass and collected from the same sites suggested genetic isolation; despite finding among the barley grass isolates some displaying spot form symptoms and some net form symptoms, the largest genetic differentiator was determined to be 'host'. This indicates that barley grass isolates contribute little to the genetics of the *P. teres* population in barley.

Results obtained from this project give insights into the *Ptm*-barley pathosystem, which can inform the development of future disease resistant barley varieties and effective fungicide application strategies. The research solidifies the importance of understanding *P. teres* genetic diversity for breeding of resistant barley cultivars and reducing reliance on fungicides. Future studies on determining the greater range and diversity of *Ptm* isolates both present and past may shed light on migration patterns and gene flow helping to determine the evolutionary potential of the disease.

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List of abbreviations and units

AFLP – Amplified Fragment Length Polymorphism

AMOVA – Analysis of Molecular Variance

CCDM – Centre for Crop and Disease Management

cv - Cultivar

CYP51A - Sterol 14-demethylase gene

DAPC – Discriminant Analysis of Principle Components

DArT – Data Array Technologies

DArTSeq – Data Array Technologies Sequencing

Fst – Fixation statistics

Gst – Nei's genetic distance

GRDC – Grains Research Development Corporation

GWAS – Genome wide association studies

ITS – Nuclear ribosomal internal transcribed spacer

MAT – Mating type locus

MLG – Multi-locus genotype

NE – Necrotrophic effector

NFNB – Net form net blotch

NSW – New South Wales

P. teres – Pyrenophora teres

PCA – Principal Components Analysis

PCR – Polymerase chain reactions

PDA – Potato dextrose agar

PhiST – F-Statistic

Ptm – Pyrenophora teres f. maculata

Ptt – Pyrenophora teres f. teres

Qld – Queensland

Qo - Quinone outside

QTL – Quantitative trait loci

RAPD – Random amplified polymorphism DNA

RSA - Republic of South Africa

SA – South Australia

SFNB – Spot form net blotch

SillicoDArT – Presence/ absence DArTSeq markers

SNP – Single Nucleotide Polymorphism

SSR – Simple sequence repeats

UPGMA – Unweighted pair group method with arithmetic mean

Vic – Victoria

WA – Western Australia

bp – base pair

cm – centimetre

°C – degrees Celsius

g – gram

kb – kilo base pair

L – litre

m – meter

µL – microlitre

µM - micromolar

ng – nanogram

s - second

List of Publications and submitted works

Publications

Hassett, K., Muria-Gonzalez, M. J., Turner, A., McLean, M. S., Wallwork, H., Martin, A., & Ellwood, S. R. (2023). Widespread genetic heterogeneity and genotypic grouping associated with fungicide resistance among barley spot form net blotch isolates in Australia. *G3 (Bethesda), 13*(5). doi:10.1093/g3journal/jkad076

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Conference attended

Hassett, K., Muria-Gonzalez, M.J., Ellwood S.R. (2022). "A Spot of Bother, transcontinental genetic diversity of *Pyrenophora teres* f. *maculata*." Participated in poster and elevated pitch presentation at the 31st Fungal Genetics Conference, March, 2022 held at Monterey Peninsula in Pacific Grove, CA (Attended online).

Contribution statement

This thesis is presented as a hybrid thesis-by-publication, containing two papers published in peer reviewed scientific journals and two additional chapters. The contribution statement provides the declarations of my contributions and those from my co-authors to each of the specified works.

Chapter 1:

Literature review, aims and objectives of the research chapters in this study.

- Substantial contributions to the conception of the chapter along with JMG, SE.
- Design of the work with assistance from JMG.
- Information acquisition and synthesis.
- Drafting of the work and revision along with JMG, SE.
- Lead writer of the chapter, with comments and suggestions from co-authors.

Chapter 2:

Widespread genetic heterogeneity and genotypic grouping associated with fungicide resistance among barley spot form net blotch isolates in Australia.

- Substantial contributions to the conception of the study along with SE and JMG.
- Designed isolate collection protocol for WA 2020 isolates along with JMG.
- Additional isolates contributed to study by SE, JMG, AT, MM and HW.
- Conducted the genotyping experiment.
- Conducted diversity and population analysis using genotypic data.
- Identified genomic regions for further analysis along with SE.
- Drafting the manuscript, with comments and suggestions from JMG, SE, AM.

Chapter 3:

Global spread, differentiation and selection of spot form net blotch.

- Conceived the design of the study with SE and JMG.
- Collected isolates for study along with WA 2021 isolates along with JMG.
- Additional isolates for study contributed by SE, JMG, AM, AK, ACO, JB, NK and RP.
- Conducted the genotyping experiment along with AM.
- Conducted diversity and population analysis using genotypic data AM, NK.
- Conceived the fungicide resistance study along with SE and JMG.
- Conducted sequencing and data analysis of isolates.
- Drafting of the manuscript and revision along with JMG, SE, AM and NK.

Chapter 4:

Population structure and genetic relationship of *Pyrenophora teres* of barley (*Hordeum vulgare*) and barley grass (*Hordeum leporinum*).

- Substantial contributions to the conception of the writing along with SE and JMG.
- Design of the work with assistance from JMG.
- Conducted the genotyping experiment.
- Conducted diversity and population analysis using genotypic data.
- Drafting of the work and revision along with JMG, SE.

I, Simon Ellwood, certify that the student's statements regarding their contribution to each of the work listed above are correct. I hereby authorise the inclusion of the co-authored work in the thesis.

Coordinating/Lead supervisor signature	Student signature
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Date:	Date:
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INTRODUCTION AND LITERATURE REVIEW

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Barley and its global status

Barley (*Hordeum vulgare*) is a versatile cereal crop with a rich history spanning millennia, serving as a testament to the intricate interrelationship between agricultural, economic, and societal influences. As a fundamental nutritional staple in numerous societies, barley has played a pivotal role in shaping civilisations, bolstering economies, and feeding populations across the globe (Badr et al., 2000). Throughout history, barley has left a permanent mark on human progress, originating in ancient agricultural systems over ten thousand years ago. Its cultivation predates many other cereal crops, making it a foundational source of food for early civilisations (Badr et al., 2000). The adaptability of barley to a range of climates and geographies has given it the status of a resilient agricultural staple, capable of thriving even in the most environmentally demanding of conditions (Lukow & McVetty, 2004). These factors have given cause to its widespread cultivation and use across diverse regions.

In ancient Egypt, barley was a principal ingredient in bread, a dietary staple for the affluent and the common people (Abdalla, 2009). In England it was also used as a unit of measurement, a medium of exchange, and a form of taxation ("Barley corn," 1769). In ancient Greece, barley-based foods such as cakes held cultural and religious importance, contributing to festivals and rituals (Foxhall, 2007). The crop's resilience to diverse climatic conditions made it a reliable food source in regions with fluctuating weather patterns, ensuring the survival of communities in challenging environments (Vanhanen et al., 2019).

In the modern world, barley maintains its significance on multiple fronts standing as the fourth largest yielding cereal crop, with an annual production of 157 million metric tonnes and covering 48 million hectares worldwide (FAOSTAT, 2020). Being an essential agricultural crop, it contributes to global food security and sustainable farming practices (Zhang & Li, 2009). Further, barley remains a key player in the brewing industry. Once fermented, it lends its unique flavour and character to the world of beer and whiskey (Craine, Bramwell, Ross, Fisk, & Murphy, 2021). Currently, its primary role lies in animal feed, which ensures the nutrition of livestock, and as an agricultural requisite contributing to global food security (Fayez & Bazaid, 2014).

Barley was introduced to Australia by European settlers in the late 18th century and was quickly adapted to the range of climatic conditions found across the continent (Lazenby & Matheson, 1975). The cereal's versatility allowed it to find a niche in various agroecological zones, from the cool temperate regions of southern Australia to the subtropical areas in the north (ABARES, 2019). Its adaptability to Australia's diverse growing conditions made it a valuable crop for Australian farmers, enabling them to diversify their crop rotations and manage risk in the face of variable climates (Lazenby & Matheson, 1975). In 1903, the first Australian barley cultivar was released, 'Prior', which was developed for the South Australian climate, quickly becoming the most popular and widely grown Australian barley variety until the late 1960s (Friedt et al., 2011).

During the late 1960's, government-supported breeding programs aided in the creation of new Australian barley varieties through the introgression of genetic material from selected cultivars across Europe, North America, Japan, and North Africa (He et al., 2022). This introgression resulted in varieties with improved yield across diverse environmental conditions, enhanced malting quality, and increased tolerance to both biotic and abiotic stressors (Friedt et al., 2011). The widespread adoption of molecular technologies such as genetic markers in the 1980's further increased the efficiency of barley breeding, allowing Australian barley breeders to introduce nearly 100 barley varieties tailored for the domestic market in the years since (He et al., 2022).

Today, barley is a staple within Australian agriculture, being the second most cultivated crop after wheat (ABARES, 2020). Furthermore, Australia is one of the world's largest barley producers and among the top three barley exporters worldwide (FAOSTAT, 2020). Currently Australia's export value is worth more than $1 billion per year in grain and malt, while global market conditions favour the Middle East as the largest market for Australian barley (Paynter, 2023a). On average Australia produces 12 million metric tonnes of barley across 4.8 million hectares with approximately 40% utilised as malting barley. Furthermore, Australian barley is highly sought after around the world for beer production and for the distillation of spirits such as malt whiskey and Japanese shochu, with the remainder being used for stock feed or human consumption in the form of bread, breakfast cereals, and other malt-based beverages (M. Gupta, Abu-Ghannam, & Gallaghar, 2010).

Globally, the successful cultivation of barley is constrained by both abiotic and biotic factors, impacting its yield and quality (Kiesling, 1985). These include abiotic stresses, such as extreme temperature, salinity and drought and biotic stresses, such as bacterial, viral, and fungal diseases. Barley diseases pose a substantial challenge to global production, necessitating effective management strategies to ensure a stable and economical supply. Some of the most economically significant diseases are fungal, including powdery mildew *(Blumeria graminis* f. *sp. hordei*), barley stripe rust (*Puccinia striiformis* f. *sp. hordei*), barley leaf rust (*Puccinia hordei*), fusarium head blight (*Fusarium spp.*), scald (*Rhynchosporium commune*) and net blotch (*Pyrenophora teres*). Net blotch is a one of the most significant disease of barley internationally (Mathre, 1997) and the most economically important disease of barley within Australia (Murray & Brennan, 2010).

Net blotch of barley

Net blotches exist in two forms, net form net blotch (NFNB) and spot form net blotch (SFNB). *Pyrenophora teres* f. *maculata* (*Ptm*), is the causal agent of SFNB of barley, whereas *Pyrenophora teres* f. *teres* (*Ptt*), the causal agent of NFNB. Both diseases are distinguished by their pathological leaf symptoms, which resemble brown necrotic lesions surrounded by a yellow halo; *Ptm*'s leaf disease symptoms differ from *Ptt*'s by the former resulting in spot-like necrotic lesions on infected leaves, which expand forming circular-shaped lesions. *Ptt*'s disease symptoms emerge similarly but expand along and across the veins of infected leaves, resulting in symptoms resembling a net (McLean, Howlett, & Hollaway, 2009) (Figure 1—1).

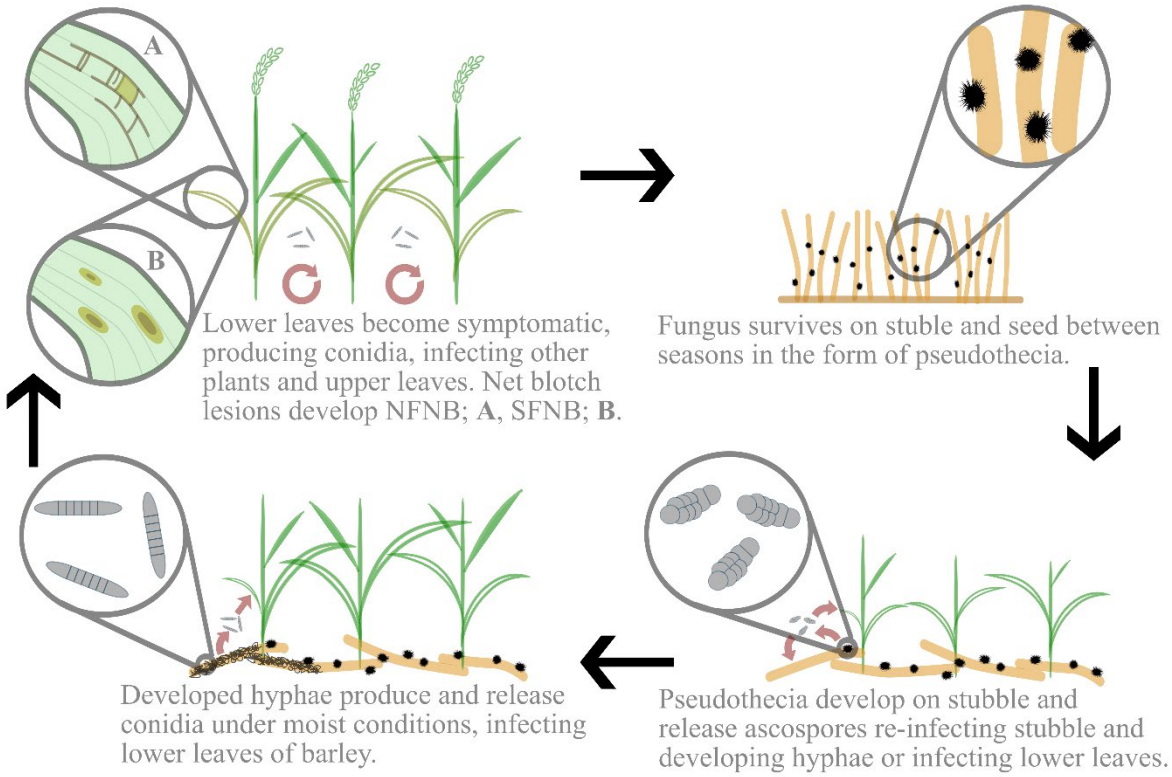

Figure 1—1 Simplified life cycle of *Pyrenophora teres* on barley. Red arrows indicate spore movement, black arrows indicate next stage in life cycle. A; symptoms typical of Net Form Net blotch (NFNB) with dark brown elongated lesions traversing leaf veins, forming net-like patterning. B: symptoms typical of Spot Form Net Blotch (SFNB) with elliptical brown spots surrounded by a chlorotic halo.

In Australia, *P. teres* has been considered an economically important foliar pathogen of barley since the 1960s, while internationally, the pathogen's disease impacts have been documented since the early 1920's (McLean et al. 2009; Shipton 1966). In 2010, the net blotch fungi were estimated to cause the greatest economic loss of all barley pathogens, averaging AUD $62 million annually (Murray & Brennan, 2010). The economic impact of the disease is primarily due to the significant reduction in yield, seed weight, grain size and downgrades in grain quality specification (Khan, 1987; Shipton, 1966). In severe outbreaks, NFNB can lead to a substantial reduction in kernel weight, ranging from 10 to 40%, and can even result in the complete destruction of the entire plant, as documented by Grewal, Rossnagel, Pozniak, and Scoles (2008) and Shjerve et al. (2014). In Australia, NFNB has been responsible for yield losses exceeding 20%, as reported by Shipton (1966), Khan (1987) and (Murray & Brennan, 2010). On the other hand, SFNB outbreaks have been associated with a seven percent reduction in grain weight (Khan, 1989) and a significant yield loss of up to 44% (Jayasena, Burgel, Tanaka, Majewski, & Loughman, 2007), with the extent varying depending on factors such as season, sowing date, and barley variety. Of these two diseases, SFNB is thought to be the most devastating, causing substantial yield and grain quality downgrades, such as kernel size, bulk density, and plumpness causing a lowered grain specification resulting in up to 40% the crop's potential economic value.

Classification of *P. teres*

Pyrenophora teres taxonomically belongs to the kingdom of Fungi, phylum Ascomycota, class Dothideomycete, order Pleosporales, family Pleosporaceae, genus *Pyrenophora*, and species *teres*. This taxonomic classification has changed over time, with, *P. teres* initially classified under the genus of *Helminthosporium*. However, later revisions were made based on the cylindrical morphological characteristics of its conidia, leading to its placement under the genus *Pyrenophora* (Shoemaker, 1959).

Pyrenophora teres was first recorded in the late 1800s (Saccardo, 1886), but the separation between *Ptt* and *Ptm* was formally proposed 90 years later (Smedegård-Petersen, 1971), existing as two genetically distinct lineages. The individual speciation has been suggested to be as a result of species divergence and specialisation on separate hosts in the past, with recent secondary contact on the same host occurring after reproductive barriers evolved (Yuzon et al., 2023).

Given that at early stages of infection, both *Pyrenophora teres* formae could be easily confused, genetic screening is often required for a more accurate identification (Liu, Ellwood, Oliver, & Friesen, 2010). In the early 2000s, Williams, Smyl, Lichon, Wong, and Wallwork (2001) employed polymerase chain reaction (PCR) to distinguish between the two formae by generating two distinct amplicons, measuring 411 bp for *Ptt* and 378 bp for *Ptm*. Further research focused on population genetics, and genetic characterisation clearly established *Ptt* and *Ptm* as genetically discrete entities, forming separate clusters (Keiper, Grcic, Capio, & Wallwork, 2008; Liu, Ellwood, et al., 2010; McLean et al., 2009; Poudel et al., 2017; Williams et al., 2001), despite occasional hybridisation between these two forms.

SFNB has been determined to be present in all major barley-growing regions globally, being first described in Australia in Western Australia (WA) in the late 1970s (Khan & Tekauz, 1982) and later identified in all other Australian barley-growing regions. Since then, SFNB has become increasingly important in Australia due to its escalating economic impact related to yield and quality losses for barley growers. Currently, all commonly grown cultivars in WA are susceptible to SFNB, apart from the feed varieties Fathom and Combat, which show moderate resistance (Paynter, 2023b). The high disease incidence is thought to be due, in part, to no-till practices, in which stubble is retained between seasons within a field, increasing the severity of residue-borne diseases in following seasons, with the pathogen overwintering in the previous harvest's crop residue, providing the primary inoculum for the next year's crop (McLean et al., 2009).

P. teres life cycle

Both *Ptt* and *Ptm* share similar life cycles (Figure 1—1) (McLean et al., 2009). Pseudothecia (fruiting bodies) surviving on barley stubble or seed form the primary inoculum of the fungus which can be seen as black sea urchin-like growths between 1-2 mm in diameter (Mathre, 1997). Both *Ptm* and *Ptt* are both heterothallic, requiring thalli from each of the two mating types (mating type I and mating type II) to produce the sexually reproductive structure (W. McDonald, 1963). Bitunicate asci then develop in the mature pseudothecia, with each ascus comprising of eight ascospores, occurring as four genetically identical pairs (Fincham, 1971), between 18 to 28 µm in length and each ascospore composed of up to two longitudinal septa and between three and four transverse septa (Mathre, 1997). Once mature and favourable conditions are met, ascospores are forcefully ejected up to 35 cm into the air and may travel long distances carried by the wind, initiating crop infection or reinfecting stubble (Backes, Guerriero, Ait Barka, & Jacquard, 2021).

Once the ejected ascospores find a suitable host plant, colonisation occurs and the asexual (anamorph) stage of *P. teres* produces conidia in groups of up to three, which develop on top of conidiophores (Mathre, 1997). Conidia are yellowish brown in colour and cylindrical, measuring

between 30 to 175 µm in length with rounded ends and the main body divided into up to 14 pseudosepta (McLean et al., 2009). Conidia produced from infected stubble serve as the primary inoculum whereas those formed on the surface of lesions of infected leaves form the secondary inoculum (McLean et al., 2009). The formation of conidia occurs within 14 to 20 days after primary infection with dispersal by wind or by rain splash (Mathre, 1997).

Infection occurs once conidia land on the leaves of a suitable host, germinating within a couple of hours under optimal conditions of temperatures between 15 and 25 °C and 100 % relative humidity (Mathre, 1997). Due to the disease's ability to undergo multiple infection-reinfection cycles during each growing season there may be heightened disease severity within fields under favourable conditions (McLean et al., 2009). The fungus survives between growing seasons in the form of pseudothecia on the surface of barley stubble, on volunteer or re-growth plants and as seed borne mycelium (Liu, Ellwood, et al., 2010; McLean et al., 2009).

Both *P. teres* forms are described as having hemibiotrophic pathogenic lifestyles, which involve a short asymptomatic or biotrophic stage in which the infection is established before a switch to necrotrophy (Caeseele & Grumbles, 1979; Lightfoot & Able, 2010). Fungal biotrophic and necrotrophic lifestyles are polar opposites accompanied by different host infection strategies. Biotrophs, which require the host cell to remain alive, establish infection by either evading or suppressing the host plant's immune system by secreting effector proteins into the host's epidermal tissue; in a susceptible interaction, the pathogen will effectively reprogram the host's cells to source nutrients while suppressing any host immune response (Koeck, Hardham, & Dodds, 2011). However, if these effectors are recognized by the plant, the plant undergoes a hypersensitive response (HR), leading to the death of host cells in the local vicinity of the pathogen's infection, hindering the biotroph from continuing infection (Glazebrook, 2005). Necrotrophs, which on the other hand require host cell death to obtain nutrients, also secrete effector proteins but differ in that they mainly secrete them into the host's apoplastic tissue; in a susceptible interaction, necrotrophs obtain nutrients from the dead host cells resulting from induced necrosis or HR-like reactions (Vleeshouwers & Oliver, 2015). Hemibiotrophs, such as the two *P. teres* forms, sit between these two pathogenic lifestyles, transitioning from biotrophy to necrotrophy once the infection is established, increasing the complexity of the pathosystem.

Variation in *P. teres* virulence

An organism's ability to produce disease can be highly variable, and can be assessed at the level of individual isolates or across populations. With regards to plant fungal diseases, commonly studied phenotypes are the degree of isolate leaf symptom development on different host genotypes, response to individual pathogen effectors, and fungicide resistance. It is also common practice to compare pathogenicity of isolates using a differential set of barley cultivars which enables them to be classified into isolate groups also known as pathotypes. Studies comparing *Ptm* isolates have shown SFNB virulence variation between countries, revealing significant differences in barley cultivar susceptibility (Khan & Tekauz, 1982), while screens of multiple *Ptm* isolates from a single populations has revealed pathotype differences within populations (S. Gupta, Loughman, D'Antuono, & Bradley, 2012).

The first reports of pathotype variation of net blotch was made in the late 1960's (Khan & Boyd, 1969). The Khan and Boyd (1969) study showed that the *Ptt* population in Western Australia at the time was virulent on the most widely grown variety, Beecher. Beecher was then replaced with the also susceptible variety, Dampier till the 1970's, at which point the NFNB-resistant variety, Clipper, was released. Interestingly, *Ptt* isolates collected between the mid-1970's and the early 1990's showed no virulence on the previously susceptible variety, Beecher (S. Gupta & Loughman, 2001; Khan & Tekauz, 1982). S. Gupta and Loughman (2001) concluded that the rapid adaptation in virulence of the *Ptt*

population was due to the switch in widespread cultivar popularity to one with NFNB resistance within Western Australia.

The phenotypic variability in NFNB barley disease was more recently evaluated in Australia, where national collection of 123 *Ptt* isolates were screened against a panel of 31 barley varieties (Fowler et al., 2017). The study identified four isolate groups, which clustered based on their capacity to infect four barley genotypes. Three of the groups were found to be present in Western Australia, and the study also found that the composition of each state's isolates generally differed based on historic cultivation practices with virulence being higher on previously widely grown cultivars (Fowler et al., 2017).

Variable SFNB pathotypes have been described in multiple countries, including Australia (Platz, Bell, Rees, & Galea, 2000), Algeria (Boungab, Belabid, Fortas, & Bayaa, 2012), Canada (Akhavan et al., 2016), Ireland (Tuohy, Jalli, Cooke, & O' Sullivan, 2006) and Turkey (Oğuz & Karakaya, 2017). Further, the pathogenic variability of *Ptm* isolates within WA has also been previously studied. The most recent study used a panel of 26 barley lines to screen 49 geographically diverse isolates collected in 2001 and 2002 (S. Gupta et al., 2012). The study identified seven pathotypes, five of which were randomly distributed through the growing regions within WA. These results aligned with the findings of S. Gupta and Loughman (2001) on the pathogenic variation of WA isolates based on their reaction on the barley line Herta. However, the work published in 2012 presented a few limitations: half of the isolates collected could not be classed into an isolate group, this may suggest that the number of isolates collected may have been insufficient to validate the pathotypes identified. Another issue is that the samples used were close to a decade old at the time of the publication. Since *Ptm* virulence is known to evolve rapidly, the isolates used may not have been representative of the current *Ptm* population. The researchers indicated that, due to the lack of grouping, some of the pathotypes may be more widespread and that further research was needed to properly elucidate the true distribution of WA's *Ptm* isolate groups.

In the Australian state of Victoria, the virulence of 44 *Ptm* isolates collected between 2003 and 2005 were assessed (McLean, Keiper, & Hollaway, 2010). In contrast to other international studies, McLean, Keiper, et al. (2010) found low pathogenic diversity in Victoria, indicating there may have been low selection pressure, given the absence of resistant cultivars. The study also found that the varieties used in the differential barley set containing the known resistance genes; *Rpt4* and *Ha4* successfully provided resistance to all tested isolates, however, due to the high genetic diversity found in Victorian isolates, it was suggested that this resistance may be quickly overcome unless properly managed. The authors also proposed that more work needed to be done to determine the pathogenic structure of other Australian states, and that there may be broader *Ptm* pathogenic diversity elsewhere in Australia (McLean, Keiper, et al., 2010).

In Western Australia, in common with to Victoria, current and historically grown cultivars are predominantly susceptible to *Ptm* (Garlinge, 2005; Paynter et al., 2020). Therefore, the hypothesis proposed by McLean, Keiper, et al. (2010), that in the absence of resistant varieties a population may lack pathotype structure may apply. This was supported by S. Gupta et al. (2012), where an inability to distinguish pathotypes in half of the isolates tested was found. Interestingly, new evidence suggests that cv Baudin, which until recently was extensively grown in Western Australia, became highly susceptible to a new pathotype capable of overcoming seedling resistance (Muria-Gonzalez et al., 2023).

Identification of pathogen virulence factors in *P.teres*

The resistance and susceptibility of barley varieties to fungal pathogens is associated with both quantitative and qualitative (gene-for-gene) traits. To determine genetic regions associated with disease traits such as resistance and susceptibility of barley varieties to *P. teres*, both qualitative and quantitative trait loci (QTL) mapping is used, which correlate the frequency of a phenotypic trait to the presence of a genetic marker. On the pathogen side, studies typically focus on effectors (most often host selective toxins), which are important in disease development and progression in susceptible barley varieties (Liu, Ellwood, et al., 2010).

Genome-Wide Association Studies (GWAS) provide a powerful means of identifying genetic regions associated with phenotypic traits, by analysing thousands or even hundreds of thousands of single nucleotide polymorphisms (SNPs) across the genome. Over the last decade or so, GWAS have transformed the field of complex disease-association genetics, providing numerous powerful associations in a wide variety of pathosystems (Tam et al., 2019). In relation to *P. teres*, GWAS have been performed to identify genomic regions or candidate genes associated with pathogenicity, virulence, fungicide resistance, and other important agronomic traits contributing to the understanding of genetic factors underlying disease development, host-pathogen interactions, and potential targets for disease management (Koladia et al., 2017). Most GWAS studies on net blotch, however, focus on the plant side of the plant-pathogen interaction such as those by Esmail, Jarquín, Börner, and Sallam (2023) and Burlakoti et al. (2017), which used GWAS on a large panel of barley cultivars to identify significant SNPs associated with resistance to NFNB and SFNB while providing insights into the genetic architecture of resistance.

Studies focusing on the pathogen side are less common, typically aimed at identifying genomic regions associated with virulence and target necrotrophic effectors (NE, Clare et al., 2022; Martin et al., 2020). In Martin et al. (2020) the researchers employed two approaches, GWAS and biparental mapping of *Ptt*. For the former, a diverse panel of isolates was genotyped, and their virulence evaluated. Several genomic regions in chromosomes three and five significantly associated with virulence were found, containing effector candidates for further investigation, and an understanding of the underlying mechanisms involved in virulence. In the bi-parental mapping approach, a mapping population was developed by crossing two isolates with contrasting virulence phenotypes, the progeny was genotyped, and their virulence traits were evaluated. The researchers identified specific genomic regions inherited from each parent isolate that were significantly associated with virulence. Combining the results from both approaches provided a comprehensive understanding of the genomic regions associated with virulences. In Clare et al. (2022) 26 novel QTL were identified using restriction-site associated DNA genotyping-by-sequencing (RAD-GBS), with the results suggesting that chromosome three of *Ptm* contains a variety of NE, similar to the findings of (Martin et al., 2020).

Genetic factors for barley resistance/ susceptibility to net blotch and pathogen virulence

Host mapping studies have identified important loci for pathogen virulence and host resistance/susceptibility using biparental and association mapping studies. To date, over 340 QTLs associated with barley responses to *Ptt*, and over 140 QTLs for *Ptm* have been published. Of these, eight specific loci, designated *Rpt1* through *Rpt8*, have been identified on barley chromosomes 3H, 1H, 2H, 7H, 6H, 5H, 4H, and 4H, respectively.

The study of barley resistance to net blotch began with Geschele in 1928, revealing quantitative inheritance of resistance to NFNB (Geschele, 1928). Later, the first single gene resistance locus, *Pt1*,

was discovered using Tifang × Atlas crosses and determined to be effective against Californian *Ptt* isolates (Schaller, 1955). Additional resistance loci, *Pt2* and *Pt3*, where then identified by Mode and Schaller (1958), noting that *Pt1* and *Pt2* were closely linked with a low recombination rate. Subsequent studies discovered the Pt_a locus to be effective against Australian *Ptt* isolate, W.A.-2, and was thought to be distinct from *Pt1*, *Pt2*, and *Pt3* loci (Khan & Boyd, 1969). Further publications resulted in the reclassification of both *Pt1* and *Pt2* loci as alleles of the *Rpt1* complex on chromosome 3H (Bockelman, Sharp, & Eslick, 1977) and later included *QRpts3L*, and *QNFNBSLR* as other alleles (Graner, Foroughi-Wehr, & Tekauz, 1996; A Lehmensiek, Platz, Mace, Poulsen, & Sutherland, 2007; Raman et al., 2003).

The Bockelman et al. (1977) study further described the presence of an incomplete dominant resistance gene to NFNB found on chromosome 1H and was designated *Rpt2c* utilising the CIho 9819 barley line. Later studies have corroborated the existence Rpt2c in CIho 9819 (Manninen et al., 2006), with a change in naming convention to *Rpt2.c* proposed by Bregitzer, Lundqvist, and Blake (2013).

The *Rpt3.d* locus was first described by Bockelman et al. (1977) on chromosome 2H (Graner et al., 1996). Interestingly, QTLs conferring NFNB resistance on multiple barley lines have implicated this locus (Cakir et al., 2003; Raman et al., 2003; Steffenson, Hayes, & Kleinhofs, 1996).

Historically, studies attempting to identify resistance loci to *Ptm* are far more infrequent than *Ptt*. More recent studies have identified three major loci, designated *Rpt4*, encompassing a 36 cM region on chromosome 7H (Wang et al., 2015; Williams et al., 1999). Furthermore, studies have suggested the broad-spectrum ability of *Rpt4*, in primarily seedling resistance to *Ptm*, finding consistent effectiveness in geographically distinct isolates (Alhashel et al., 2021; Daba, Horsley, Brueggeman, Chao, & Mohammadi, 2019; Tamang et al., 2019).

Research involving barley *Ptm* pathogenicity has identified two major virulence loci on *Ptm* chromosomes 1 and 2. The virulence locus on *Ptm* chromosome 2 targets a dominant susceptibility gene at the *Rph4* locus on barley chromosome 7H (Skiba et al., 2022). Skiba et al. (2022) further demonstrated the likelihood of an inverse gene-for-gene relationship in the barley-*Ptm* pathosystem, highlighting the complex interactions between host and pathogen genetics.

The *Rpt5* (*Spt1*) locus found on from CIho 9819 on chromosome 6H has been extensively studied in the *Ptt*-barley interaction and is thought to be under complex control (Abu Qamar et al., 2008; Cakir et al., 2003; Manninen et al., 2006). Differential isolate susceptibility towards *Ptt* isolates has been found in Kombar and Rika barley varieties being conversely susceptible to *Ptt* isolates 15A and 6A respectively (Abu Qamar et al., 2008). Abu Qamar et al. (2008) also identified two alleles, *rpt5.k* and *rpt5.r* mapped to approximately 5.9 cM region, as recessive resistance genes due to a 1:3 resistance segregation ratio, requiring homozygosity for resistance. Further refinement of the region by Liu, Faris, Edwards, and Friesen (2010) refined this region to about 1.6 cM suggesting the *rpt.k /rpt.r* region is near the centromere on the long arm of chromosome 6H.

The *Rpt6* locus was first found in CI9819 on the short arm of chromosome 5H conferring resistance to both *Ptm* and *Ptt* (Burlakoti et al., 2017; Manninen et al., 2006). Manninen et al. (2006) utilised biparental mapping of Rolfi × CI 9819, finding *Rpt6* explained <84% of the observed disease variation.

Rpt7 was first found in in Halcyon, located on the long arm of chromosome 4H, conferring resistance to *Ptt* (Raman et al., 2003). Many other studies using other barley varieties have identified similarly localised QTLs, however it is currently unclear whether they represent the same locus (Clare, Wyatt, Brueggeman, & Friesen, 2020). A second unrelated locus designated *Rpt7* was later found to confer resistance to *Ptm*, however, this locus was found on chromosome 7H and likely represents a previously undesignated locus found by Williams et al. (2003) (Tamang, Neupane, Mamidi, Friesen, & Brueggeman, 2015).

Rpt8 was first identified in on chromosome 4H conferring resistance to *Ptm* (Friesen, Faris, Lai, & Steffenson, 2006). This QTL and all others discussed are thought to provide a valuable resource for breeding broad-spectrum resistance to SFNB and NFNB. However, the identity and functional mechanisms of the underlying genes remain elusive.

Population genetics and genetic diversity of *P. teres*

Developing an understanding of the genetic structure of a pathogen population is key to developing resistance-breeding strategies and improved disease management (B. McDonald & Linde, 2002). These rely on knowledge of pathogen genetic distribution in time and space, the use of representative isolates for genetic screening, and the discovery of genotype by host interactions (Burdon, 1993). Further, over-time, continuous exposure to selective pressures such as resistance genes and fungicides leads to the eventual breakdown of these control mechanisms (B. McDonald & Linde, 2002).

The genetic structure and diversity of a population is defined by the pattern of the genetic makeup of its individuals. This results from mutation, gene flow, recombination, genetic drift, bottlenecks and selection (B. McDonald & Linde, 2002). Genetic diversity is important to establish population substructure, which in turn may inform whether the release of developed disease resistant cultivars is sustainable and durable. When breading for plant resistance, the use of representative isolates of the target pathogen population to guide the selection is necessary, and controlled exposure to all different pathotypes where the plant will be deployed is required to ensure that the resistance will be effective in the field. Since pathogen evolution often occurs rapidly in response to deployment of new host resistance genes, regular monitoring following the introduction of new genotypes is needed to monitor the emergence of new pathotypes.

Pathogen genotype-by-host genotype interactions impact the rate at which a pathogen population overcomes resistance. There are two main types of host resistance: race-specific resistance is effective against isolates carrying a corresponding avirulence gene and is commonly overcome by selection for mutations in the pathogen that avoid recognition, leading to a defeated resistance gene, whereas partial resistance tends to be effective against all isolates in the pathogen population (Pilet-Nayel et al., 2017). Therefore, identifying whether resistance is partial or race-specific is necessary for predicting the durability of resistance genes.

Occurrence and population structure of *P. teres*

The cultivation of barley spans a spectrum of climates worldwide, and both forms of *P. teres* are known to cause outbreaks in all barley-growing regions (van den Berg, 1988). Nevertheless, the most prevalent type of *P. teres* can vary both between countries and regions over-time (Arabi, Al-Safadi, & Charbaji, 2003; Mäkelä, 1972). For instance, in Europe, NFNB epidemics are more pronounced in the widely grown susceptible spring barley varieties, whereas the *Ptm* is known to develop higher levels of infection in winter barley varieties (Minarikova & Polisenska, 1999). In Australia, temporal shifts in the population dynamics of net blotch have led to changes in the dominant form of the disease across various regions (McLean, Keiper, et al., 2010). Historically, *Ptt* has been implicated as the primary causative agent of net blotch (Hirsch, 1988; Khan & Tekauz, 1982). However in recent years there has been an increased prevalence of SFNB, this is thought to have arisen due to the increased cultivation of susceptible barley varieties (McLean, Howlett, & Hollaway, 2010).

Early genetic studies utilised a various methods of generating molecular markers, including random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLPs) and simple sequence repeats (SSRs), These studies have reported the occurrence of high genetic diversity within the *P. teres* populations collections internationally, including Australia (Bogacki, Keiper, & Oldach, 2010; A. Lehmensiek et al., 2010; McLean et al., 2014; Serenius, Manninen, Wallwork, & Williams, 2007), Europe (Bakonyi & Justesen, 2007; Rau et al., 2003), Iran (Vasighzadeh et al., 2021), northern

Africa (Ahmed Lhadj et al., 2022), North America (Akhavan et al., 2016), the Republic of South Africa (RSA) (Campbell, Lucas, & Crous, 2002; A. Lehmensiek et al., 2010) and Turkey (Çelik Oğuz & Karakaya, 2021).

The genetic structure of the *Ptm* populations has been assessed both Australia-wide and by state (Bogacki et al., 2010; McLean, Keiper, et al., 2010; McLean et al., 2014). McLean et al. (2010) conducted both a genetic and pathotype analyses of Victorian isolates with genetic diversity examined using 15 polymorphic simple sequence repeats. There was a high level of genetic diversity, contrasting the absence of pathogenic diversity described for the state's *Ptm* population (McLean, Keiper, et al., 2010). This is consistent with other international studies on *Ptm* genetic diversity where there has been a lack of correlation between genotypic and pathogenic diversity (Duellman, 2015; Serenius et al., 2007). Literature on other fungal species suggests that this lack of correlation may be due to the evolutionary rate of the pathogen genome compared to the evolutionary rate of individual genes, meaning that genetic changes occur frequently which may not affect gene structure or function (Chen, Line, & Leung, 1993). Another factor is that not all genetic variation within a species leads to effects of on pathogenic variation, as these effects may be found in non-coding regions or relate to non-pathogenicity associated loci.

Other studies on *Ptm* in Australia generally concluded that there is a high degree of genetic relatedness between *Ptm* populations within Australia, with sexual reproduction occurring within fields (Bogacki et al., 2010; McLean, Keiper, et al., 2010), however there is likely population structure unrelated to region of isolation (McLean et al., 2014). International studies tend to support these observations (Akhavan et al., 2016; Campbell et al., 2002). Since 2014, there has been an absence of studies into the population structure and genetic diversity of Australian *Ptm* isolates.

Conventional marker systems, such as AFLP and RAPD, have limitations when it comes to data reproducibility. New marker systems have emerged, offering a significantly greater amount of genome-wide molecular markers, with some being situated within gene regions. One such high-throughput molecular marker technology is Diversity Arrays Technology (DArT), which possesses the advantage of not requiring the previously sequence genomes (Wenzl et al., 2004). DArTseq™ represents a cost-effective and innovative advancement over the original DArT methodology, leveraging Next Generation Sequencing (NGS) techniques for the use of genotyping-by-sequencing. This technology introduces polymorphisms at recognition sites of restriction enzymes and detects the presence or absence of individual DNA fragments in the genome via fluorescently labelled microarray tags (Jaccoud, Peng, Feinstein, & Kilian, 2001). DArTseq™ yields a higher number of individually identifiable markers (Kilian et al., 2012) and has been tailored for use with fungal species, including *P. teres* (Syme et al., 2018).

The most recent population structure and genetic diversity study conducted using DArTSeq markers on *P. teres* was published in 2021 (Dahanayaka et al., 2021). This study investigated the international distribution and local genetic adaptations of 250 *Ptt* isolates collected from Australia, Canada, Hungary, and Republic of South Africa (RSA), as well as historical isolates from Canada, Denmark, Japan, and Sweden using 5,890 DArT markers. The study revealed global population sub-structuring through the implementation of modern data analysis techniques. One such technique included the Discriminant analysis of principal components (DAPC), revealing four genetic clusters of isolates. Two of these clusters contained isolates from all regions, and all isolates from the RSA were grouped in these two. Australia and Hungary were present in three clusters each. This suggested that the global population structure of the pathogen is likely poorly defined by sample region and as isolates from different regions were genetically similar, and multiple instances of global migration may have caused the resulting genetic heterogeneity. The study also found genetic markers associated with population

structure near genes related to fungal virulence, pathogenicity and fungicide resistance including *GPD1*, *CYP51A* and a non-ribosomal peptide synthetases.

Disease management

Net blotch management encompasses various strategies, including the utilisation of resistant cultivars, fungicide applications, the control of alternative host plants, and the elimination of primary sources of infection through crop rotation and stubble destruction (Liu, Ellwood, et al., 2010). Crop rotation is considered one of the most effective practices, and requires growers to refrain from cultivating barley in the same field year-on-year by introducing instead non-host crops of *P. teres* (Rees, 1999). Repeated barley cultivation in the same field, coupled with the use of the same barley variety, can increase pathotype selection due to accelerated adaptation towards host resistance genes. Further, persistent stubble retention can harbor disease inoculum from barley crops grown in previous years (Jordan & Allen, 1984).

The use of resistant varieties is another method of disease management, given that the survival of the different pathotypes depends on several environmental influences, namely the host barley variety (Jonsson, Säll, Kraft, & Gustafsson, 1999). Further, the overreliance on susceptible barley varieties not only escalates the frequency of outbreaks but also amplifies the disease's severity (McLean, Keiper, et al., 2010). Therefore, a concerted effort to cultivate resistant barley varieties is thought to be the most practical, and long-term solution for effective disease management (Mathre, 1997).

However, the severity of net blotch symptoms on barley genotypes may vary depending on the pathogenic diversity of *P. teres* isolates contained within a given population (Liu, Ellwood, et al., 2010). Where, pathogenic diversity is defined by the disease population's ability to infect a range relevant crop varieties or cultivars often using a range of virulence mechanisms. In the case of SFNB the disease appears to be characterised by the prevalence of quantitative interactions presented as a continuum of responses on the host plant, rather than the more discrete symptoms associated with qualitative resistance or susceptibility (Burlakoti et al., 2017).

In Australia, the pathogenic diversity of commercially relevant fungal pathogens, including *P. teres*, is monitored annually by challenging commercially relevant barley varieties against different pathotypes (Table 1-1) (Paynter, 2023b). Disease ratings are determined by assessing seedling and adult stage foliar disease severity. This assists growers in choosing suitable barley varieties based on past experiences with disease severity in their region.

Table 1-1. List of popular and relevant cultivars and net blotch susceptibility scores.

Popular cultivars [a]	Release date [b]	Usage [c]	Popularity [d]	SFNB [e]	NFNB [f]
Maximus CL	2018	Malt	32%	MSS	S - MS
Spartacus CL	2014	Malt	30%	SVS	MS - MRMS
RGT Planet	2016	Malt	12%	S	S - MS
Rosalind	2014	Feed	7%	S	MS - MR
Buff	2016	Malt	3%	S	MS - MRS
La Trobe	2013	Feed	2%	S	MS - MRMS
Scope CL	2008	Feed	2%	S	S - MRMS
Flinders	2007	Malt	2%	S	S - MRMS
Commodus CL	2020	Stage 2	2%	MSS	MRMS - MSS
Fathom	2011	Feed	1%	MRMS	MSS - SVS
Beast	2019	Stage 2	2%	MSS	MRMS - MSS
Litmus	2013	Feed	1%	MS	S - MSS
New cultivars					
Cyclops	2020	Stage 2	N/A	MSS	MRMS - MSS
Laperouse	2016	Stage 2	N/A	MSS	MRMS - MSS
Minotaur	2020	Stage 2	N/A	S	MS - MRMS
Combat	2021	Feed	N/A	MRMS	MS- MSS

[a] Commercial name of respective cultivar.

[b] Release year of the respective cultivar in Australia.

[c] Relative popularity (percentage of barley area) of the top ten barley varieties delivered in WA in 2023. The top ten varieties occupied 96% of the area planted to barley in the season (Paynter & Khan, 2024).

[d] Current usage designation as of 2024, varieties classed as malt have been accredited by the Barley Council of Grains Australia. Varieties classed as Stage 1 or 2 are under evaluation for their malting and brewing end-use.

[e,f] Average resistance profile of cultivar to disease R, resistant; RMR, resistant to moderately resistant; MR, moderately resistant; MRMS, moderately resistant to moderately susceptible; MS, moderately susceptible; MSS, moderately susceptible to susceptible; S, susceptible; SVS, susceptible to very susceptible; VS, very susceptible. Data obtained and adapted from information presented by Paynter (2023b); (Paynter & Khan, 2024).

Due to the lack of resistant barley varieties, the most frequently used method of disease control for NB is fungicide application (Sierotzki et al., 2007). Both foliar and seed treatment is currently used as a method of control for NB. *Ptm* is however the costliest pathogen of barley to manage in Australia, costing $43 million annually (Murray & Brennan, 2010).

There a three primary classes of fungicides that are used to control net blotch internationally, with quinone outside inhibitors (QoI ; FRAC group 11), succinate dehydrogenase inhibitors (SDHI; FRAC group 7), and demethylase inhibitors (DMI; FRAC group 3) currently being the most common (APVMA, 2019; Ireland, 2021; Wesley J. Mair et al., 2016). Reports of resistance to DMI fungicides are becoming frequent, and resistance to QoI fungicides, such as azoxystrobin and pyraclostrobin, has also been reported, although it appears to be less common (Knight, Adhikari, Dodhia, Mair, & Lopez-Ruiz, 2023; Wesley J. Mair et al., 2020; Poudel et al., 2017).

Fungicide resistance

There have been reports of both *Ptm* and *Ptt* developing resistance to fungicides, which is a concern for growers and agronomists as it can limit the effectiveness of chemical control measures (Knight et al., 2023). To minimise the risk of fungicide resistance, it is important to follow best management practices, such as rotating different fungicide modes of action, using fungicides only when necessary, and using integrated pest management strategies incorporating cultural practices, resistant cultivars, and biological control agents (GRDC, 2018; Liu, Ellwood, et al., 2010). Currently in Australia, the most commonly used fungicides for controlling SFNB are DMIs, QoIs and SDHIs (Ireland, 2021) but evolutions in resistance towards these fungicides has been observed, affecting their efficacy and performance (Lammari, Rehfus, Stammler, & Benslimane, 2020; Wesley J. Mair et al., 2016).

Demethylase inhibitors (DMI) fungicides have a precise target site, the cytochrome P450 lanosterol 14/ CYP51/ sterol 14α-demethylase enzyme (Lamb, Kelly, & Kelly, 1999). This fungicide class exhibits selectivity by binding to the active site of CYP51, inhibiting the biosynthesis of ergosterol, a critical component of the cell membrane (Berg, Plempel, Buchel, Holmwood, & Stroech, 1988). QoI fungicides provide biotic control over the pathogen by impeding mitochondrial respiration; when these fungicides successfully bind to the Quinone outside (Qo) site within the cytochrome bc1 complex, interrupting the electron transport chain within the mitochondria (Bartlett et al., 2002) halting the synthesis of ATP, ultimately inhibiting spore germination and the development of mycelia (Bartlett et al., 2002). SDHI fungicides also affect fungal respiration by targeting the succinate dehydrogenase enzyme, a pivotal enzyme connecting the tricarboxylic acid cycle to the electron transport chain (Fernández-Ortuño, Torés, de Vicente, & Pérez-García, 2008).

Currently, all classes of commonly used fungicides have concerns over their effectiveness against *P. teres*. This is thought to be due to the widespread usage of fungicides with the same mode of action, putting evolutionary pressure on the pathogen to develop mechanisms of resistance.

The first instance of DMI insensitivity in NB populations was reported in the early 1980s (Sheridan, Grbavac, & Sheridan, 1985). However, there has been an increase in reported incidences in recent years, as many global populations of *P. teres* have developed resistance to newer variations of azole compounds, such as tebuconazole and epoxiconazole (Campbell & Crous, 2002; Wesley J. Mair et al., 2016). Reduced resistance to DMIs from both *Ptm* and *Ptt* isolates collected from Western Australia is reportedly as a result of the overexpression of the *Cyp51A* gene due to a 134 bp transposon insertion in the promoter, which, in combination with F489L mutations leads to highly resistant isolates (Knight et al., 2023; Wesley J. Mair et al., 2016; Wesley J. Mair et al., 2020). F489L mutations alter the target site of the fungicide, reducing the binding affinity of tebuconazole and rendering it less effective against resistant strains. The emergence of tebuconazole-resistant strains has significant implications for NB management, as it limits the efficacy of tebuconazole-based fungicides in controlling the disease. Consequently, alternative fungicides or integrated disease management strategies need to be implemented. Studies to determine the evolution and movement of fungicide resistance in *P. teres* globally thus far have not been conducted.

The QoI class fungicides resistance has been reported in central Europe since 2003 (Sierotzki et al., 2007) and later in the UK in 2007 (Marzani, 2013). Mutations leading to resistance exist in two target cytochrome b site mutations, F129L and G137R (Semar, Strobel, Koch, Klappach, & Stammler, 2007). Currently these fungicides remain effective in Australia with no confirmed cases of the cytochrome b mutations.

The first recorded instance of lower sensitivity to SDHIs in NB was in Germany in 2012 (Stammler, 2013). These isolates carried a H277Y substitution in the succinate dehydrogenase enzyme B subunit, and in the following two years, ten other gene mutations related to resistance in the C and D subunits

were reported. These amino acid substitutions affect the binding affinity of SDHI fungicides within the ubiquinone-binding pocket formed by the B, C and D subunits (Stammler, Wolf, Glaettli, & Klappach, 2015). Since then, nearly half of all isolates collected in France and Germany contain one of the mutations (Rehfus et al., 2016), with later reports suggesting similar mechanisms of resistance being present in Australia (W.J. Mair et al., 2023).

Alternative hosts of *P. teres*

Pyrenophora teres is known to infect a variety of plant species beyond cultivated barley. Among these hosts are wild grasses in the genus *Hordeum*, including barley grass *(H. murinum L.),* wild barley *(H. spontaneum (K. Koch)),* bulbous barley *(H. bulbosum L.),* and other cereal crops such as wheat (*Triticum aestivum*) and oat (*Avena sativa*) (van den Berg, 1988). These alternative host plants often coexist with cultivated barley, and can serve as potential sources of inoculum for *P. teres*, contributing to its disease spread (Linde & Smith, 2019b). This broader host range stresses the importance of understanding the pathogen's interactions with various plant species and its potential impact on crop health and management strategies.

Barley grass (*H. lemporium* (Link) Arc), also commonly known as wall barley, a subspecies of *H. murinum*, is an annual weed that grows alongside cereal crops such as cultivated barley in growing regions within Australia, acting as a source of inoculum of *P. teres* (McLean et al., 2009; Preston, 2019). Although considered a sister species to barley, bulbous barley, and wild barley, it is thought to have separated from them 8 Mya, compared to the 9.2 Mya from which all other *Hordeum* species groups diverged (Brassac & Blattner, 2015). Barley grass is an introduced weed to Australia that grows predominantly within the southern cereal-growing regions, often found alongside and within commercial barley fields. In Australia, the weed performs as a 'green bridge' to the virulent fungal root diseases Take-All in wheat (Preston, 2019) and the foliar leaf disease, scald, in cultivated barley (Linde, Smith, & Peakall, 2016).

Wild barley (*H. spontaneum*) is currently considered the progenitor and a subspecies of *H. vulgare* and is thought to have originated from southeast Asia (Blattner, 2018; von Bothmer & Jacobsen, 1985). Of the genus *Hordeum*, wild barley is considered the closest relative to domesticated barley, separating some ten thousand years ago (Blattner, 2018). Wild barley, which is primarily present in the Levant, Turkey, and East of Turkey, is a host to *P. teres* isolates similar to those found in *H. vulgare* (Çelik Oğuz, Ölmez, & Karakaya, 2019). This makes wild barley a reservoir for diseases with different genetic adaptations and pathogenic variation.

Bulbous barley (*H. bulbosum* L.) is also considered a close sister species of *H. vulgare*, capable of producing fertile hybrids, and with a similar distribution to wild barley (Brassac & Blattner, 2015). Further, bulbous barley is considered to have split from barley and wild barley approximately 3.7 Mya (Blattner, 2018). Despite this, both forms of *P. teres*, *Ptm* and *Ptt*, show a high degree of pathogenic similarity to isolates found in *H. vulgare*, capable of infecting barley (Çelik Oğuz et al., 2019).

Host specificity refers to the degree to which a particular organism, such as a parasite, pathogen, or symbiont, is adapted to infect or associate with a specific host species or a narrow range of host species. In the context of plant pathogens, like viruses, bacteria, fungi, or parasites, host specificity determines which organisms or variants of a pathogen can infect and the severity of the resulting disease. Host specificity alongside genetic relatedness of *P. teres* found on barley grass remains a topic of debate in Australia. Some studies suggest that economically relevant forms of *P. teres*, *Ptt*, and/or *Ptm* can infect species of *Hordeum* without specificity (Bakke, 1912; Braverman, 1960), whereas other studies have suggested inconclusive results (Brown, Steffenson, & Webster, 1993), or inferred strict host specificity (Khan, 1973; Linde & Smith, 2019a).

Other inconsistencies in the current literature are the lack of clarity behind the specific symptomatic forms of *P. teres* found on barley grass. Older literature has typically focused on net form or did not specify a singular symptomatic form/ type. This was also the case in a recent study, indicating that the net form lesions that were observed on barley grass, implied the pathogen form/s on barley grass were more related to NFNB than SFNB on *H. vulgare* (Linde & Smith, 2019a).

Knowledge gaps and research opportunities

This review identifies key aspects related to the *Pyrenophora teres*-host interaction that have been underexplored or insufficiently addressed in the current body of literature. This establishes the foundation for the subsequent research endeavours presented in this thesis, aiming to contribute novel insights and fill these voids in our scholarly understanding:

Understand the current population structure and genetic diversity of Ptm in Australia and discover genomic regions associated with population substructure.

Genetic characterisation of Australian *Ptm* populations is crucial for comprehending regional variations. Despite the importance of SFNB, no recent studies have been carried out and none at the level of genome-wide diversity. Existing literature, mainly based on AFLP and microsatellite techniques, lacks the resolution for moderate-low genetically diverse populations, which may be required in the case of this pathogen. This research addresses these gaps by employing the DArTseq™ genome-wide marker system, enabling the identification of key genomic regions associated with Australia's *Ptm* population genetic sub-structuring. This approach facilitates a more in-depth exploration of the migration patterns and phenotypic traits in *Ptm* populations. The study sets the stage for comprehensive analyses of *Ptm* genetics, enhancing our insights into diversity and migration.

Understand the population structure and genetic diversity of Ptm populations from different continents and investigate the potential source of fungicide resistance in Western Australia.

Aligned with the previous objective, I wanted to understand better the global genetic variation in *Ptm* populations. Previous studies, mainly involving single countries, in Australia, Europe, Iran, northern Africa, North America, the Republic of South Africa, and Turkey relied on conventional markers like SSR, RFLP, or RAPD. However, large-scale multi-regional genomic studies comparing *Ptm* diversity are lacking. The third chapter of this thesis incorporates isolates from Australia, Hungary, the Republic of South Africa, Turkey, Canada, and Denmark. This approach aims to reveal regional and continental genetic relationships, migration patterns, and population structures of *Ptm*, and contributed to a deeper understanding of fungicide resistance origins in WA.

Investigate the genetic relationships of P. teres of barley grass and its foliar pathogenic forms and study their relationship with P. teres forms on barley.

The genetic relationship between forms of *P. teres* in barley and those from barley grass, along with their host specificity, lacks formal exploration using modern whole-genome GBS markers. The third experimental chapter of this thesis addresses these issues, investigating diversity on barley and barley grass in lower Western Australian growing regions. Using 119 *P. teres* isolates, the study determines regional genetic relationships, host-associated pathogenicity, and symptomatic form genetic relationships between separate hosts. This research provides an up-to-date overview, enhancing our understanding of *P. teres* population biology and genetic relationships in Australia.

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WIDESPREAD GENETIC HETEROGENEITY AND GENOTYPIC GROUPING ASSOCIATED WITH FUNGICIDE RESISTANCE AMONG BARLEY SPOT FORM NET BLOTCH ISOLATES IN AUSTRALIA

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Abstract

Spot form net blotch, caused by *Pyrenophora teres* f. *maculata*, is a major foliar disease of barley worldwide. Knowledge of the pathogen's genetic diversity and population structure is critical for a better understanding of inherent evolutionary capacity and for the development of sustainable disease management strategies. Genome-wide, single nucleotide polymorphism data of 254 Australian isolates revealed genotypic diversity and an absence of population structure, either between states, or between fields and cultivars in different agro-ecological zones. This indicates there is little geographical isolation or cultivar directional selection and that the pathogen is highly mobile across the continent. However, two cryptic genotypic groups were found only in Western Australia, predominantly associated with genes involved in fungicide resistance. The findings in this study are discussed in the context of current cultivar resistance and the pathogen's adaptive potential.

Introduction

Spot form net blotch (SFNB) is a major foliar disease of barley worldwide (Liu, Ellwood, Oliver, & Friesen, 2011), caused by the ascomycete fungus *Pyrenophora teres* f. *maculata* (*Ptm*) Smed.-Pet. (1971). The pathogen is morphologically similar, yet phylogenetically distinct from *Pyrenophora teres* f. *teres* (*Ptt*), the causal agent of net form net blotch (NFNB) disease. The two diseases are distinguished by their physiological leaf symptoms, with SFNB characterised by round brown lesions surrounded by a yellow chlorotic halo and NFNB characterised by dark brown, net-like necrotic lesions striate along barley leaf veins. Despite occurring on the same host and their similar morphological characteristics, the two diseases are treated separately as they interact with different host resistance and susceptibility genes (Clare, Wyatt, Brueggeman, & Friesen, 2020) and, although natural hybridisation is possible, this is rare (Poudel et al., 2017). In Australia, SFNB is of economic importance, with yield losses of up to 20% and an increase of up to 18% in undersized grain (McLean, Poole, Santa, & Hollaway, 2022).

Fungal pathogen evolution in plants is broadly governed by effectors, small rapidly evolving secreted proteins or molecules which interact with the host in different ways dependent on pathogen lifestyle (Plissonneau et al., 2017). For example, biotrophs secrete effectors that supress host defences and subvert metabolism to allow proliferation (Dodds et al., 2009). Necrotrophs, by contrast, secrete effectors that promote cell death (Faris & Friesen, 2020). Host recognition and effector diversification drive the interaction between fungal pathogens and plants, with dominant host resistance against biotrophs leading to the loss or alteration of the corresponding virulence (effector) gene. In necrotrophs such as *Ptm*, there is a predominantly opposite genetic relationship, known as the inverse gene-for-gene model, where susceptibility is dominant as genes encoding host target products are lost or altered (Muria-Gonzalez et al., 2023; Peters Haugrud, Zhang, Richards, Friesen, & Faris, 2019).

The genetic structure and diversity of *Ptm* has been previously assessed within Australia using both SSRs (simple sequence repeats, Bogacki, Keiper, & Oldach, 2010; M. McLean, F. Keiper, & G. Hollaway, 2010; M. McLean, J. Keiper, & G. Hollaway, 2010) and amplified length polymorphism (AFLP) markers (Lehmensiek et al., 2010; McLean et al., 2014; Serenius, Manninen, Wallwork, & Williams, 2007). These studies analysed modest numbers of both isolates (31 – 60) and polymorphic markers (15 - 109) and found low genetic differentiation between different regions and fields and no correlation with geographic origin, although both Serenius *et al.* (2007) and McLean *et al.* (2010) found differences in the levels of genetic diversity between regions.

Globally, genetic diversity studies of *Ptm* have been performed in Europe (Bakonyi & Justesen, 2007; Rau et al., 2003), North America (Akhavan et al., 2016), Algeria (Ahmed Lhadj et al., 2022), Iran (Vasighzadeh et al., 2021), the Republic of South Africa (Campbell, Lucas, & Crous, 2002), and Turkey (Oğuz, Ölmez, & Karakaya, 2019). In common with previous Australian studies, these employed older genetic marker technologies including SSRs, ISSRs (inter-simple sequence repeats) and AFLPs and generally found significant levels of sexual recombination and low levels of clonality. The studies in both Sardinia and Canada found no significant genetic differentiation between populations. In Iran, however, using the same SSRs as those in the Canadian study, higher genetic variation was apparent together with significant evidence for regional population structure, while Lehmensiek *et al*. (2010) were able to distinguish isolates from South Africa and Australia.

Although microsatellites and AFLP DNA markers have been commonly used for comparing individuals with high levels of genetic diversity, they usually lack high marker numbers (in the case of microsatellites) and precise genomic context and even genomic distribution (in the case of AFLPs) available with genotype-by-sequencing (GBS) techniques, where large, single nucleotide polymorphism (SNP) datasets are produced. GBS techniques have been shown to provide clearer detection of finer scale genetic structuring compared to microsatellite data (Sunde, Yıldırım, Tibblin,

& Forsman, 2020). Furthermore, hundreds or thousands of SNP markers generated throughout the target genome produce higher resolution data on smaller sample sizes compared to SSRs (Jeffries et al., 2016). This allows better comparisons between both strongly and weakly diverged populations (Andrews, Good, Miller, Luikart, & Hohenlohe, 2016), as well as better inferences on population structure (Bruneaux et al., 2013). One popular method of generating SNP markers is by Diversity Arrays Technology (DArT), a highly parallel genome-wide approach based on a restriction enzyme complexity reduction step that selects for non-repetitive (coding) DNA (Sansaloni et al., 2011). The DArT system produces reproducible silicoDArT (presence-absence markers) and DArTseq (SNP) markers from genomic DNA extracts. This technology has been previously implemented for genetic mapping and genotypic analyses of fungal species including *P. teres* (Poudel et al., 2019; Syme et al., 2018).

We hypothesised the composition of the *Ptm* population across Australia is likely to be influenced by geographic isolation and the cultivars being grown, and that low sample numbers together with few or potentially clustered genetic markers in previous studies may have excluded genomic regions contributing to population structure. To assess this, and to provide greater resolution of the genetic diversity and the distribution of *Ptm* genotypes in Australia, we analysed isolates based on DArTseq SNP markers. These were sampled at two levels; on the one hand isolates collected from across the major barley growing regions and, on the other hand, isolates collected from six fields at three sites in Western Australia. The isolates were collected between 2017 and 2020, with the majority of Western Australian isolates collected in 2020. Their genetic diversity was then assessed and the relationships between isolates was compared by Bayesian, multivariate and geographic distance-based approaches.

Methods

Field-scale *Ptm* isolate collections

Isolates were sampled from six fields across three agricultural zones (Agzones) of Western Australia (WA) for inter-field analyses (Table S2, Figure 2—1). Agzones 2, 3 and 6 were chosen due to their status as high rainfall zones (>450 mm per year) suited to barley growth, whilst spanning the majority of the barley growing area of WA. Two fields per Agzone were sampled, one of barley cultivar (cv) Spartacus CL and the other of cv RGT Planet. In each field, samples were obtained from a 60 m x 100 m area divided into 100 m^2 sections. Three leaves displaying SFNB symptoms were collected from each section and stored in paper envelopes.

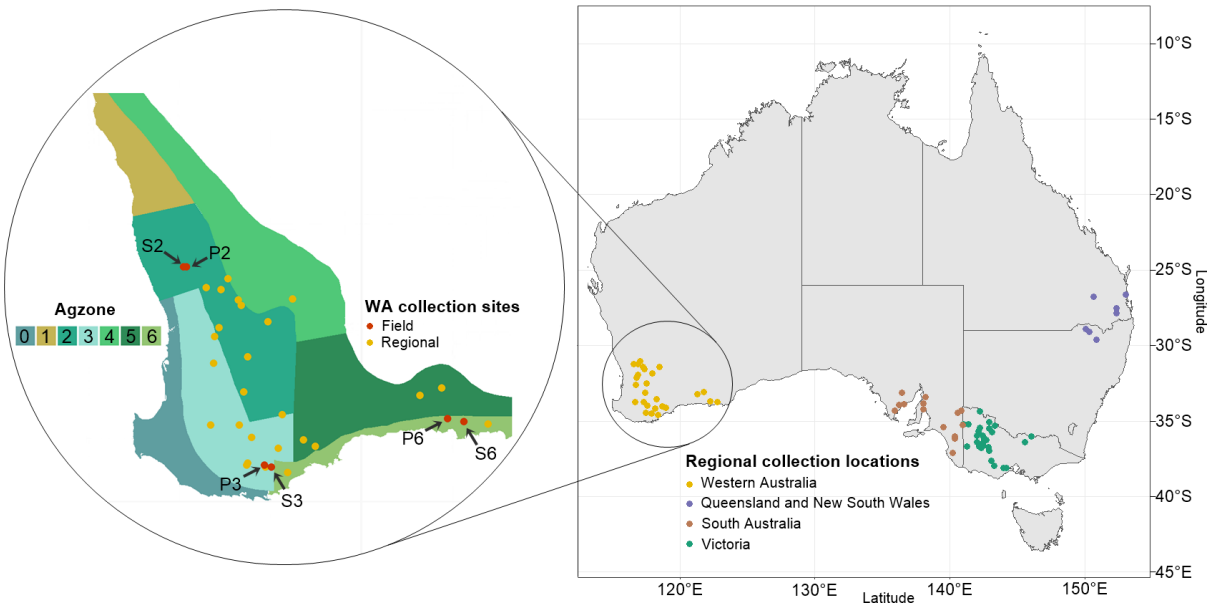

Figure 2—1. *Ptm* collection sites from Australian states and Western Australian Agzones. Red dots indicated by arrows represent six Western Australian fields from which diseased leaves were sampled. Yellow dots represent sampling locations for the regional Western Australian Ptm isolates, brown dots for the South Australian isolates, green dots for the Victorian isolates and purple dots for the isolates collected from southern Queensland and northern New South Wales. Agzone map data obtained from Geographic Information Services, Perth, WA (2016).

Inter-regional collections of *Ptm* isolates and related *Pyrenophora* sp.

To investigate national *Ptm* diversity, a total of 87 *Ptm* isolates were provided by the Centre of Crop and Disease Management (CCDM), the South Australian Research and Development Institute (SARDI) and Agriculture Victoria (AgVic, Figure 2—1). The isolates were from locations dispersed across the majority of major barley growing regions of WA, South Australia (SA), Victoria (Vic), New South Wales (NSW) and Queensland (Qld). Included in the regional *Ptm* collections were four randomly chosen *Ptt* isolates from the same institutions.

Fungal isolation

To obtain single-spored isolates, leaf samples were dried at room temperature for two weeks, then surface sterilised (30 seconds in 15 % ethanol, followed by 30 seconds in 5 % ethanol and 1 % bleach, and rinsed two times for 30 seconds in sterile water). Net blotch-like lesions were excised and placed on a petri dish containing sterile paper towel wetted with sterile water, sealed with Parafilm (Bemis Inc., Neenah, WI, USA) and incubated at 18 °C with a 14 hr photoperiod for up to seven days. Leaves were inspected for conidia formation daily under a binocular microscope from the third day post plating. Cultures where no conidia were produced after seven days were placed under near UV light for 18 hrs at room temperature, followed by 24 hrs in the dark at 15 °C to stimulate sporulation. Mature conidia were collected with a sterile acupuncture needle and transferred to V8-PDA agar plates (150 ml/l V8 juice (Campbell's Soups Australia, Lemnos, VIC, Australia), 10 g/l agar (Oxoid Ltd., Basingstoke, UK), 10 g/l Difco™ potato dextrose agar (Becton Dickinson, Sparks, MD, USA) and 3 g/l CaCO3). Plates were incubated at room temperature for five days before 4 mm^2 plugs were cut from each colony, then air-dried in a biosafety cabinet overnight before storage at -80 °C.

DNA extraction and identification of *P. teres* mating type loci

Cultures were grown for five days in Fries 2 liquid medium (Fries, 1938). The mycelia were lyophilised and genomic DNA isolated using a Promega Wizard Genomic DNA Purification Kit (Fitchburg, Wisconsin, USA) according to the 'Isolating genomic DNA from plant tissue' protocol. DNA concentration and quality were measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific, MA, USA). *MAT* locus mating type and *formae* were determined by the polymerase chain reaction (PCR) method according to Lu *et al*. (2010).

Genotype-by-sequencing and DArTseq data filtering

Pyrenophora teres isolates were genotyped using DArTSeq™ whole genome sequencing at Diversity Arrays Technology Pty Ltd (Canberra, Australia). DArT is a highly parallel, genome-wide genotyping technology developed by Jaccoud *et al*. (2001) combined with next-generation sequencing (Sansaloni et al., 2011) and proprietary analytical pipelines (Ren et al., 2015). DArTSeq™ genome complexity reduction was performed with *Pst*I and *Mse*I restrictionn enzymes, followed by next-generation sequencing using a HiSeq2000 DNA sequencing platform (Illumina, USA). In total 5110 DArTSeq™ (single nucleotide polymorphism (SNP) markers) and 6321 silicoDArT (presence/ absence markers) were produced. DArT genotype data is provided in Table S1.

SNP markers were converted into the Genalex file format (Peakall & Smouse, 2012) and imported into RStudio 4.2.0 (RStudio, Boston, Massachusetts, USA). Markers and isolates with >10% missing data were removed. Phylogenetically uninformative loci, those containing less than a given percentage of divergent individuals (cut off = 2/n) and minimum allele frequency (MAF = 0.01), were removed using the *informloci* command in the *poppr* package (Kamvar, Tabima, & Grünwald, 2014).

Unique multi-locus genotypes (MLGs) were determined by first establishing the genetic distance between two sets of replicate samples to compensate for SNP genotyping errors associated with DArTseq. The average genotyping error for DArTseq SNPs between pairs of template control DNA samples has been reported as averaging 0.8%, which is lower than other SNP genotyping platforms and DNA sequencing (Ndjiondjop et al., 2018). Genetic distances within both sets of biological replicates were calculated using the *provesti.dist* model, and used to filter the data with the *mlgfilter* command in the *poppr* package (default parameters), such that isolates sharing a genetic distance below the calculated value were considered the same MLG. Isolates within the same MLG were classified as clones using the *clonecorrect* function in *poppr*.

Resolution of *P. teres* forms

Based on initial comparisons, DArTSeq SNP markers (rather than dominant silicoDArT markers) were chosen to assess the *formae* relationships, together with subsequent regional and field level analyses, due to the silicoDArT markers not adding novel information. SillicoDArT markers were analysed separately, and generally supported the conclusions found with the DArT SNP markers Table S1. An initial assessment of the effectiveness of DArT genotype data to distinguish *P. teres formae* isolates was conducted using a group of four *Ptt* isolates, including reference genome isolate W1-1, the *Ptm* reference isolate SG1 (Syme et al., 2018), and four randomly selected *Ptm* isolates (106/16A, 20P3001, PTM18-024 and 71/17, see Table S2).

Genetic diversity and linkage disequilibrium

The genetic diversity of groups of isolates was determined by comparing the number of MLGs to the expected number of MLGs (eMLG) in the original data, together with Simpson's corrected index ((N/(N - 1)) λ) of multi-locus genotype diversity. To determine the extent of random mating occurring within populations, gametic equilibrium was calculated using the standardised index of association ($\bar{r}_d$), which is sample size independent (Agapow & Burt, 2001), within the *poppr* package in Rstudio (RStudio, Boston, Massachusetts, USA).

Analysis of molecular variance

In order to detect genetic variation within and between groups of isolates for each comparison (between forms, regions and fields), an analysis of molecular variance (AMOVA) was carried out. Variance (σ), as well as the population differentiation statistics (φ) were calculated using the *poppr.amova* command in the *poppr* package.

Principle components analysis and discriminant analysis of principle components

Principal component analysis (PCA), a multivariate statistical approach, was used to inform genetic clustering of individuals. In addition, genetic subdivision was inferred through discriminant analysis of principle components (DAPC), a method optimised for large datasets that like PCA does not rely on pre-existing genetic models but which can examine more complicated scenarios, detecting between-group variability and structures existing among clusters (Jombart, Devillard, & Balloux, 2010). For the PCAs, clone-corrected data was investigated with the *glpca* function in the *adegenet* R package to observe the impact of eigenvalues on the overall variance explained (Jombart, 2008). DAPC was performed after first observing the K-means clustering graph at the lowest Bayesian information criteria (BIC), using the *find.clusters* command in the *adegenet* package, and then applying the appropriate value for each of the DAPC analyses. DAPC was performed with the *dapc* command in *adegenet*, applying the result derived from the cross validation function *Xval.dapc* to confirm the correct number of PC to retain (Dray & Dufour, 2007). The *snpzip* command using the "median" method in the *adegenet* package was then used to calculate the contribution (loadings) of each SNP to groups assigned in the clustering analysis. DArTSeq sequences (69 bp in length) responsible for genetic clusters were examined by BLAST at the National Center for Biotechnology Information (NCBI) and in Geneious 8.0.5 (Kearse et al., 2012) to find annotated homologous genes in *Ptm* or in related fungi including *Ptt* and *Pyrenophora tritici-repentis* (*Ptr*) (Table S3).

Bayesian inference-based clustering analysis

Genetic structure was also examined via a Bayesian inference based method, implemented in STRUCTURE version 2.3.4 (Pritchard, Stephens, & Donnelly, 2000). STRUCTURE is a model-based clustering approach that assumes loci within populations are at Hardy-Weinberg equilibrium and linkage equilibrium, although allowing for admixture linkage disequilibrium within a population, enabling detection of subtle population subdivisions (Falush, Stephens, & Pritchard, 2003). SNP data was analysed using a burn-in period of 100000 steps and 100000 replications, assessing K values between one and nine with 10 iterations. The STUCTURE output file data was then processed with STRUCTURE HARVESTER (Earl & vonHoldt, 2011) to determine the optimal K value.

Genetic isolation by distance

To detect the correlation between genetic distance and geographic distance a Mantel test was carried out based on each isolate's sampling GPS coordinates or, in the absence of these, the closest known location. The *provesti.dist* function was used to produce a matrix of the genetic distances between individuals and used alongside the *dist* function in R to produce a distance matrix of the GPS coordinates. The *mantel.rtest* was used to calculate the likelihood of isolation by distance in the *ade4* package.

Results

Inter-*formae* SNP diversity and differentiation

In order to assess the accuracy of the DArTseq SNP dataset to differentiate the *Pyrenophora teres* forms and their relative genetic relatedness, subsamples of *Ptm* and *Ptt* individuals were compared. After data filtering, 1594 SNP markers remained. Total SNP variance within *forma* was relatively low (7.7 %), comprising only a small portion of the total observed variation, whereas variation between *formae* was high (92.3 %) and comprised the majority of the total variation. This was complimented by analysis of molecular variance (AMOVA) differentiation statistics between the two groups which was high ($\varphi = 0.92$), while a PCA showed the first two principle components explained ~90% of the variation.

Field-level population structure of *Ptm* isolates

After filtering by allele call rate and missing data, 164 out of the initial 166 WA *Ptm* isolates remained, providing 11 to 36 isolates per field. Removing non-informative loci left 1252 SNP markers for further analysis. Total SNP variance within fields was high and comprised most of the total variation (≥99%) whereas variation between fields comprised ≤1% of the total. The isolates showed a high level of individual variation, grouping into 155 MLGs (Table 2-1), while population differentiation was low ($\varphi = 0.0035$). Nei's unbiased gene diversity, which indicates the probability that two randomly chosen alleles are different, was moderate for all populations (> 0.196); and the corrected Simpson's index, which represents the probability of two random isolates drawn from a population to be of a different multi-locus genotype, suggested high genotype diversity for all populations ($1-\lambda d > 0.99$). Despite the overall high genotype diversity, all fields appeared to be primarily reproducing asexually as suggested by the standardised index of association ($\bar{r}_d$) values (between 0.003 and 0.019, $p = 0.01$).

Table 2-1. Genetic diversity indices of Australian *Ptm* isolates. Data is presented for groups sampled within WA fields and across four regional areas, produced within the poppr package (Kamvar et al., 2014).

	n	**MLG**	**eMLG**	***H***	**1-λ**	H_{exp}	$\bar{r}_d$
Field[#]							
P2	27	27	11	3.3	1.00	0.21	1.5×10^{-2}
P3	36	36	11	3.58	1.00	0.20	3.0×10^{-3}
P6	11	11	11	2.4	1.00	0.20	5.0×10^{-3}
S2	28	26	10.7	3.23	0.99	0.20	1.1×10^{-2}
S3	27	27	11	3.3	1.00	0.20	3.0×10^{-3}
S6	35	32	10.7	3.42	0.99	0.20	1.9×10^{-2}
Total	164	155*	10.9	5	1.00	0.21	8.8×10^{-3}
Region							
Vic	30	30	10	3.4	1.00	0.203	9.20×10^{-4}
SA	20	20	10	3.00	1.00	0.208	1.52×10^{-3}
Qld and NSW	8	8	8	2.08	1.00	0.19	3.38×10^{-3}
WA	29	27	9.7	3.25	0.99	0.208	9.16×10^{-3}
Total	87	85	9.97	4.43	1.00	0.211	2.52×10^{-3}

#: Indicates host (P for RGT Planet or S for Spartacus CL), with agricultural zone number.

n: Number of isolates in a sample group after data quality filtering.

MLG: The total number of unique multi-locus genotypes (MLGs) identified per field or region. *Indicates the cumulative number of MLGs irrespective of field or region.

eMLG: Expected number of MLGs.

H: Shannon-Wiener index of MLG group genotypic diversity, a measure of the number of unique genotypes and their homogeneity.

1- λ: Corrected Simpson's index of MLG diversity, the probability two isolates from the same dataset are different genotypes.

H_{exp}: Nei's unbiased gene diversity index, the probability that two randomly selected alleles are different.

$\bar{r}_d$: The standardised index of association, with a value of zero for a null hypothesis a population is freely recombining.

A rarefaction curve showed no significant deviation in the number of MLGs found within each field based on non-clone corrected data (Supplemental Figure 1), while a lack of curve saturation suggested a large number of MLGs exist at each sample site. Four MLGs contained between two and six individuals, with the remaining 151 MLGs consisting of a single representative. MLG 49 was the largest and most geographically widespread group, detected in three fields and comprising six individuals (Figure 2—2).

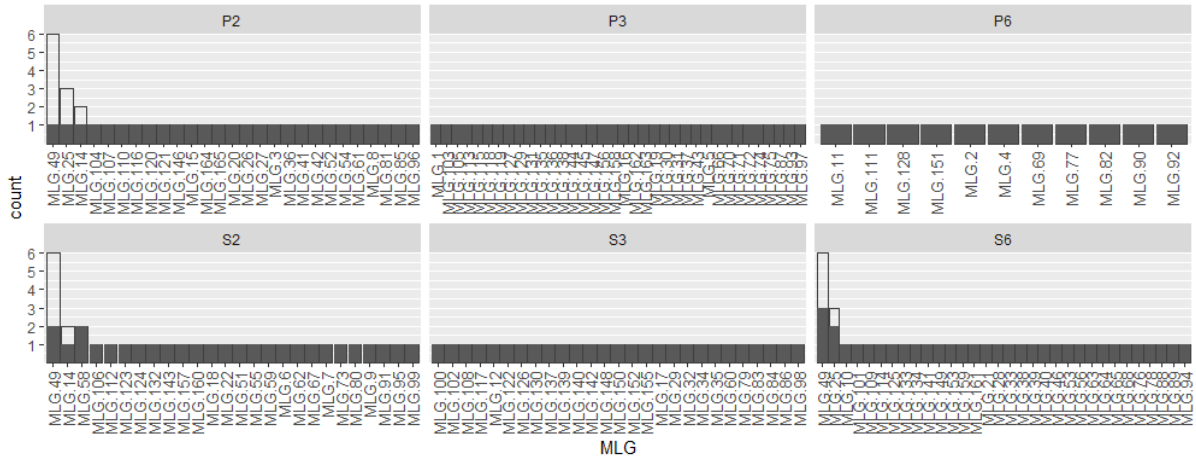

Figure 2—2. Distribution and size of *Ptm* MLGs across six fields within Western Australia. Number of individuals per MLG per field (black columns) is shown compared to the total observed individuals per MLGs over the six-field sample set (black outlined column).

Principal components analysis showed no observable population structure when the first two principal components were plotted against one another (Figure 2—3). However, unsupervised clustering analysis performed without *a priori* knowledge of sample location suggested two potential populations (K = 2) when 150 principal components were retained. A membership probability plot of each of the fields illustrating the predicted populations suggested weak geographic ties to the predicted clusters (Supplemental Figure 2), while all six fields contained isolates from cluster one and four contained isolates from cluster two.

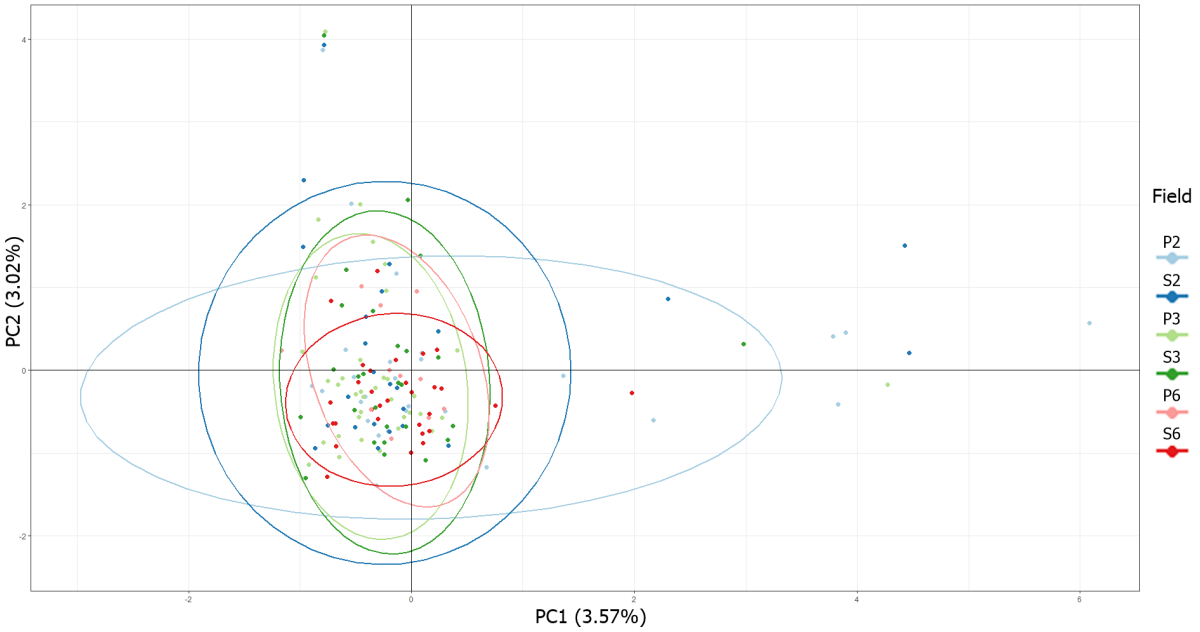

Figure 2—3. PCA of *Ptm* genotypes from WA field sampling showing the first two principal components. Isolates were collected from three different Agzones and two different host cultivars. Blue indicates isolates from Agzone 2, green isolates from Agzone 3, and red isolates from Agzone 6. Light shades of each colour indicate RGT Planet as the host cultivar while the darker shades indicate Spartacus CL. Coloured ellipses show respective 95% confidence intervals.

Further analysis of these two putative populations by AMOVA revealed variation within clusters to be high (~78%), however, variation between clusters was also moderately high (~22%). A principle components analysis was performed on the putative two populations and showed significant differentiation (Figure 2—4). The corrected Simpson's index also suggested high genetic diversity within each cluster (1-λd >0.98).

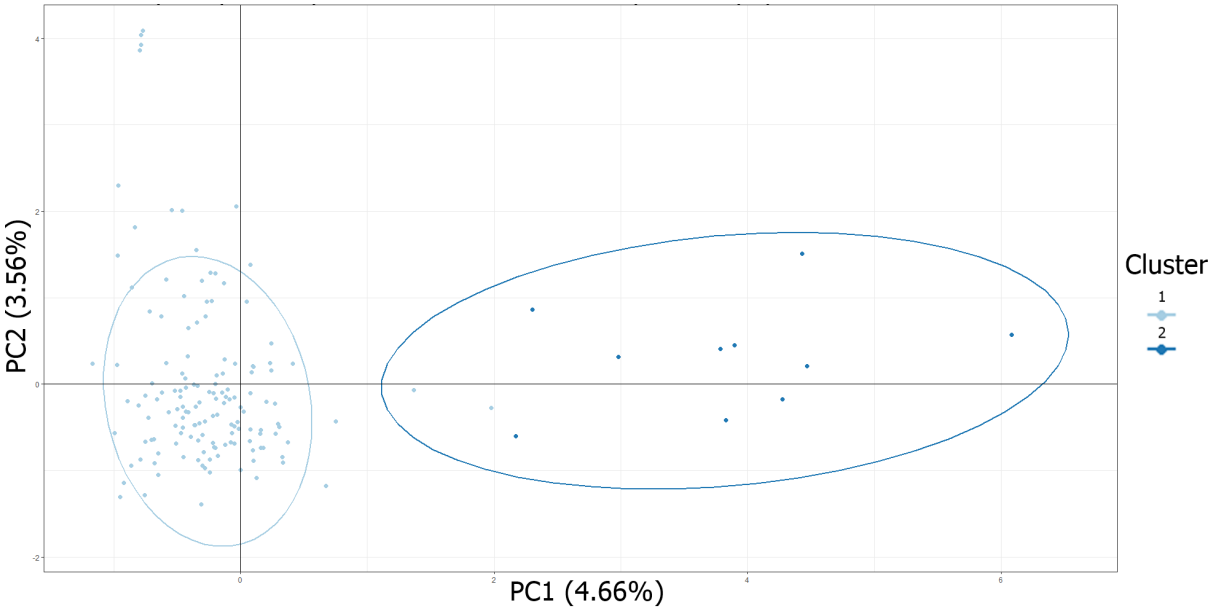

Figure 2—4. Principal component analysis of *Ptm* putative populations identified by DAPC of isolates collected from individual WA fields. The first two principle components showing differentiation of isolates depicted by light blue and dark blue dots. The 95% population assignment confidence intervals are indicated by respectively coloured ellipses.

The role of Agzone origin or isolate host variety in genetic structure was also examined. Similar to the analysis at field level, variances within Agzones and within the same host cultivar were high and comprised the majority of the total variation (99%), whereas variation between Agzone and host cultivar was low (1%). MLGs containing multiple individuals were also shared between each Agzone and cultivar.

Inter-regional population structure analysis

Isolates collected for inter-regional comparisons were placed into four groups represented by between 8 and 30 individuals based on their origin of collection: Vic, SA, WA, with Qld and NSW combined. The isolates from NSW (n = 3) and Qld (n = 5) were combined as they represent a contiguous barley growing area straddling the state border. After clone correction, SNP variance within regions was high and accounted for most of the variation (~99.1%), whereas variation between the groups was low (~0.9%). Regional differentiation of isolates was low, similar to that found at the field level (φ = ~0.01). Isolates were highly heterogeneous at the regional level, presenting 85 MLGs based on 1278 loci with each MLG confined to a single region (Table 2-1). Nei's unbiased gene diversity was high for all regions (0.19 – 0.211) and the corrected Simpson's index suggested high genotype diversity (1-λd >0.99). Similar to comparisons between groups of isolates collected within fields, each of the four regional groups appeared to be primarily asexually reproducing as portrayed by the $\bar{r}_d$ distribution.

PCA eigenvalues calculated for the first two PCAs linking samples to their sample locations accounted for a low ~6 % of the explained variation, with no visible population structure when plotted (Figure 2—5). Unsupervised clustering analysis performed without *a priori* data of sample location suggested the existence a unique population in Australia (K = 1). The Mantel test revealed a small, but positive linear correlation between genetic distance and isolate sample location (*p-value* = 0.024 and r = 0.073), suggesting that despite no detectable genetic structure being present between Australian regions, there is likely a component of isolation by distance (Supplemental Figure 3).

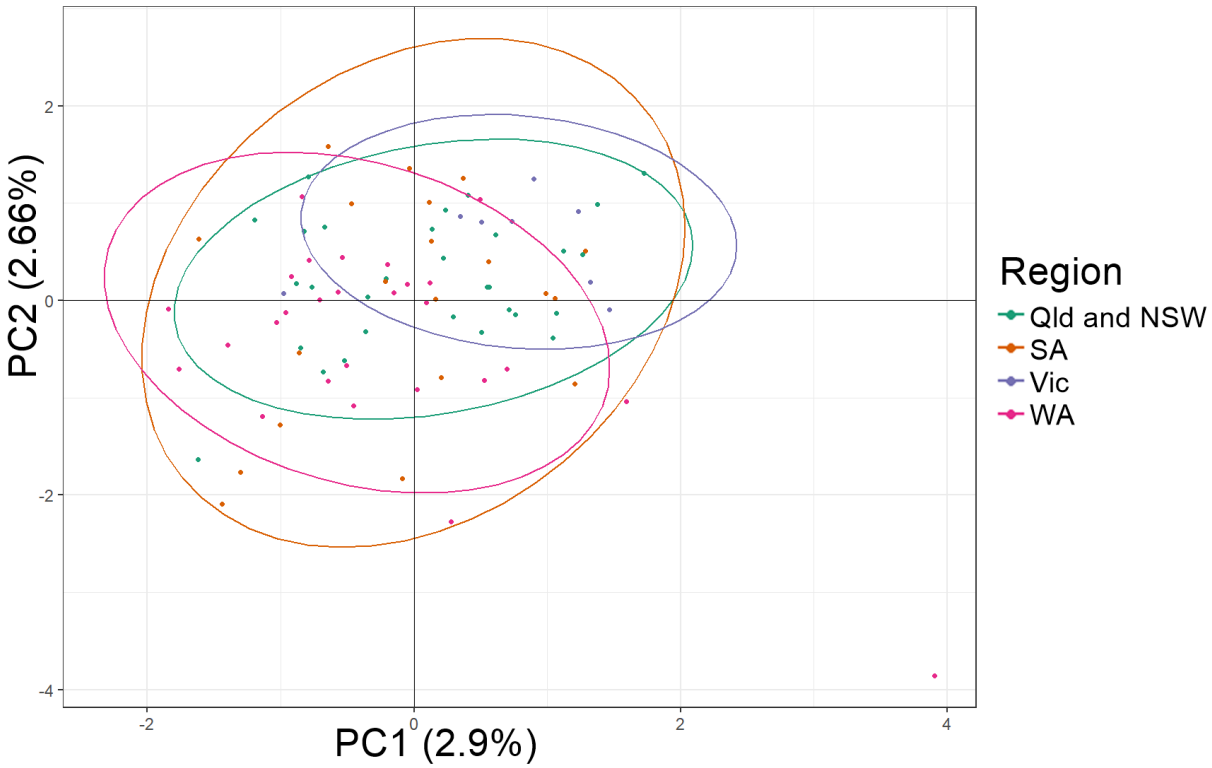

Figure 2—5. Principal component analysis of all *Ptm* samples from four Australian regions. The national groups are represented by purple, orange, green and pink dots, Respective coloured eclipses indicate 95% confidence intervals.

To increase the data resolution supporting the putative populations established with DAPC in the field level analysis, isolates from both the field level and regional analysis were combined. For this, 239 clone corrected *Ptm* isolates and 1271 filtered SNP markers were used, in order to show the total MLG genetic diversity of the four sample regions (Table 2-2). Genotype diversity was high within each of four national *Ptm* groups and similar to the previous analyses, most of the variation (~98.3%) occurred within regions whereas variation between regions was low (~1.6%), with variations between fields contributing least to genetic variation (~0.1%).

Table 2-2. Genetic diversity indices of 239 *Ptm* multi-locus genotypes collected from across Australia and of their putative DAPC genotypic groups. Data is presented for combined regional *Ptm* collections and of two putative genotypic clusters defined by DAPC, produced within the poppr package (Kamvar et al., 2014).

	MLG	*H*	1-λ	H_{exp}	$\bar{r}_d$
Region					
Vic	30	3.4	1.00	0.208	$1x10^{-3}$
SA	20	3.0	1.00	0.204	$2x10^{-3}$
Qld and NSW	8	2.08	1.00	0.192	$2x10^{-3}$
WA	181	5.2	1.00	0.208	$6x10^{-3}$
Total	239	5.48	1.00	0.209	$5x10^{-3}$
Cluster					
pop1	227	5.42	1.00	0.203	$2.54\ x10^{-3}$
pop2	12	2.48	1.00	0.194	$2.77\ x10^{-3}$
Total	239	5.48	1.00	0.208	$4.95\ x10^{-3}$

MLG: The total number of unique multi-locus genotypes (MLGs) identified per region or genotypic group.

H: Shannon-Wiener index of MLG group genotypic diversity, a measure of the number of unique genotypes and their homogeneity.

1- λ: Corrected Simpson's index of MLG diversity, the probability two isolates from the same dataset are different genotypes.

H_{exp}: Nei's unbiased gene diversity index, the probability that two randomly selected alleles are different.

$\bar{r}_d$: The standardised index of association, with a value of zero for a null hypothesis a population is freely recombining.

Unsupervised clustering analysis by PCA performed without *a priori* knowledge of sample location suggested two potential populations (K = 2) (Supplemental Figure 4). DAPC was used to study the two putative populations with thirty-two PCA eigenvalues and three discriminant analysis (DA) eigenvalues being retained (Supplemental Figure 5). Notably, one of the populations (pop1) was present in all four regions; however, WA was the only region to contain the second population (pop2). Only one Western Australian isolate from the original regional analysis (19PTX147) contributed to the pop2, the other 11 were isolates from the field level analyses. Genetic diversity indices were high between each putative population in Table 2-2. Further analysis of the pop1 and pop2 groupings by AMOVA revealed variation within each to be high (~79%), and the variation between moderately high (~21%).

Validation of the two cryptic sub-groups with STRUCTURE version 2.3.4 (Pritchard et al., 2000) generally supported the DAPC findings, with both analyses showing no geographic ties of the isolates

to their regions (Supplemental Figure 6). However, STRUCTURE indicated the presence of three potential populations rather than the two proposed by DAPC (Supplemental Figure 7). This third subpopulation of just eight isolates (population assignment >50%) consisted of much more highly related individuals of the larger population identified in DAPC, which was also present only in WA (Supplemental Figure 8).

Mating type equilibria

To determine the level of sexual reproduction of *Ptm* in Australia, mating type analyses were performed. PCR amplification of *MAT* loci of the 87 interstate *Ptm* isolates revealed 42 that were *MAT*1-1 and 45 that were *MAT*1-2. Both mating types were present in each of the four state regions did not significantly deviate from a 1:1 ratio for a randomly crossing population in both the original and clone-corrected data (chi-square tests suggested no significant deviation from an expected 1:1 ratio at $p > 0.05$). Similarly, the isolates collected from individual WA fields did not significantly deviate from the 1:1 ratio, both within and between fields, in both the unaltered and clone corrected data. The clonal isolates in the largest group of clones in MLG: 49, identified among WA isolates, congruently displayed the same mating type, *MAT*1-2 and, as may be expected, all clones within other MLGs amplified the same mating type.

SillicoDArT markers support lack of population structure and high genotypic diversity in Australia.

Dominant SillicoDArT markers were examined independently to DArTSeq SNP markers in complimentary analyses of all regional and field *Ptm* isolates (Supplemental analysis.). Results generally agreed with those based on the SNP markers. The SillicoDArT markers distinguished the same MLGs and supported the genetic diversity estimates, with most of the genetic variation (~98%) occurring within regions whereas variation between regions was low (~1.4%), while variation between fields contributed least to genetic variation (~0.6%). The silicoDArT markers also indicated high genotypic diversity but isolates were closely related.

Unsupervised clustering analysis primarily supported the SNP data, which established a group of isolates clustering separately from the other MLGs (Figure 2—4: Cluster 2, Table 2-2: pop2). Notably, the same WA genotypic group was observed. Clustering analysis however differed in suggesting the existence of three other cryptic subpopulations unrelated to region, Agzone field, or host variety.

Population differentiating SNP markers are located in genomic regions associated with fungicide resistance.

Following DAPC of all clone-corrected isolates, 18 unique markers were associated with differentiation of the two SNP-based genetic clusters (Table S2). All the markers mapped by BLASTn to *Ptm* isolate SG1 (BioProject: PRJEB18107, BioSample: SAMEA4560037) within Geneious 8.0.5 (Kearse et al., 2012) with E-Values ranging from 3.92×10^{-30} to 4.04×10^{-10} with >95% identical sites shared. Of the 18 markers, 11 mapped to candidate genes and seven mapped to intergenic regions. The most differentiated marker was found at ~8500bp from the fungicide resistance associated gene *Cyp51A* (GenBank accession: CP060577). Two other markers were also found within 16000bp and 31000bp of this gene. Two markers were found near to a *Ptr* homologue for a Major Facilitator Superfamily (MFS) gene annotated as a benomyl/ methotrexate resistance protein (GenBank accession: XM_001931024).

Orthologous genes in *Ptr* to the 11 SG1 genic markers included a calcium channel protein (GenBank accession: XM_001931060), a tRNA A64-2'-O-ribosylphosphate transferase protein (GenBank accession: XM_001931591), a pumilio domain containing protein (GenBank accession: XM_001931022), low homology to a HC-toxin producing non-ribosomal protein synthase (GenBank

accession: XM_001940729), together with other unnamed predicted proteins or conserved hypothetical proteins.

Discussion

This study combines the largest and most geographically diverse Australian collection of *Ptm* isolates to date with a high-resolution genetic marker technology, enabling an improved resolution of the genetic diversity and relatedness in the pathogen population. Overall, the study confirms high levels of genotype diversity and low levels of clonality. Similar results in both forms of *P. teres* have been observed previously in Australia (Bogacki et al., 2010; Ellwood et al., 2019) and in other countries such as Sweden (Jonsson, Sail, & Bryngelsson, 2000), Italy (Rau et al., 2003), Czech Republic (Leisova, Kucera, & Minarikova, 2005), Finland (Serenius et al., 2007), Canada (Akhavan et al., 2016) and Iran (Vasighzadeh et al., 2021). Modest levels of genetic recombination was observed both within fields and within national regions. This was shown through linkage disequilibrium analysis using the standardised index of association, $\bar{r}_d$. For a freely recombining population, the expected score is zero and greater than zero if there in an association between alleles. Both categories had scores greater than zero, indicating limited sexual reproduction with asexual propagation the primary mode. However, equal mating type ratios and low numbers of clonal isolates were also observed, suggesting sexual reproduction is frequent enough to maintain mating type balance.

Lack of geographic population structure

Isolation by distance and physical barriers to migration commonly contribute to genetic differentiation between individuals. However, despite Australia being some 4000 km wide, geographic clustering of genotypes was absent in this study, with low variation between regions. Wind dispersal of spores provides the most likely explanation. However, the Nullarbor Plain is a mostly treeless, arid area, which stretches over 1000km and separates WA from the rest of the barley growing regions in Australia. Despite this, clustering analysis indicated a single Australian population (K = 1). Over larger distances, assisted dispersal in infected straw and hay may play a role in supressing population differentiation, as these are commonly transported between states, particularly during droughts (*Ptm* is not regarded as a significant seed borne pathogen). Another contributing reason may be due to the diseases' relatively recent introduction into Australia, being first identified in Nabawa, WA in 1977 (Khan & Tekauz, 1982), and a lack of time enabling divergence from a founder population. This is a less parsimonious explanation, and assumes similar genotypic lineages have established and persisted throughout the country. Nonetheless, despite the lack of obvious geographic population structure, a Mantel test suggested some isolation by distance, although the effect was low.

Analysis of the population differentiation between fields in WA provided similar results to regional comparisons, with low genetic differentiation between individuals from different Agzones, fields, and the host variety, but a high degree within. This may be expected as, unlike comparisons of genotypes between different regions defined by clear geographic boundaries, there are extensive barley growing regions in WA from Agzone 2 through to Agzone 6. Furthermore, the distribution of MLG groups indicated neither Agzone nor host cultivar appeared to play a role in genotypic selection among the isolates. The cultivars the isolates were sampled from, RGT Planet and Spartacus CL, were independently developed by different breeding companies. Both are rated as susceptible to SFNB and the *Ptm* genotypic compositions on these hosts do not indicate independent gain of virulence. These have been the most popular varieties sown in WA in recent years, whereby Spartacus CL was the most common variety in 2019 and 2020 with RGT Planet in second position, together accounting for nearly 70% of the area sown (Paynter, 2023). As other popular cultivars are predominantly susceptible, the detection of gain of virulence by *Ptm* in Australia might be regarded as the exception. However, anecdotal reports suggest *Ptm* is capable of defeating SFNB resistance. For example in WA, Thomas *et*

al. (2018) noted the erosion of partial seedling resistance in cvs Scope and Hindmarsh, while seedling resistance was overcome in cv Baudin (Muria-Gonzalez et al., 2023).

In the most intensively sampled region, WA, just three MLGs were found with individuals distributed across sites used for field level sampling at locations over 650 km apart. The low number of clones in these MLGs (eleven) provides weak evidence for recent selection of beneficial genotypes, perhaps masked by susceptible popular cultivars. For example, predominantly clonal *Ptm* isolates were previously found in cv Oxford in southern WA, associated with a virulent new pathotype in combination with DMI fungicide resistance (Turo et al., 2021). The transience of the genetic background of clonal expansions in *Ptm* may be similar to *Ptt*, where DMI resistance was rapidly assimilated into the wider population (Ellwood et al., 2019).

Genotypic clustering is independent of geographic origin

Clustering analysis by DAPC suggested the presence of two populations (K = 2). These were not linked to the field of origin, with individuals present across most of the sampled fields, potentially suggesting a recent introduction or selection of new genotypes variants in WA. Bayesian analysis with STRUCTURE suggested the presence of three populations (K=3). The two populations identified by DAPC were supported, with a third comprised of more highly related individuals representing a subset of the larger DPAC pop1 group. This population was present only in WA and may represent selection of an advantageous genotype, and contains MLG: 49, the largest group of clones found in WA. However, despite the removal of isolates sharing the same MLG prior to STRUCTURE analysis, the validity of this group should be assessed in the context of the limitations of the STRUCTURE algorithm, which requires populations to be in Hardy-Weinberg equilibrium.

A lack of Australian *Ptm* geographic population structure is in agreement with a study by McLean et al. (2014), which assessed the genetic structure of *Ptm* in Australia using a geographically diverse set of isolates collected between 1996 to 2009 (n = 60). The authors also identified two genetically distinct *Ptm* clusters unlinked to sampling year and sampling region, however, in that study an Australia-wide population structure was not established. Our study confirms the presence of a single Australia-wide *Ptm* population, with one or more smaller populations unrelated to sampling site, referred to here as cryptic populations.

Our results also complement aspects of other *Ptm* genetic diversity studies conducted within Australia. Bogacki *et al.* (2010) found that isolates sampled from different areas of the same field and between fields were genetically similar and Serenius *et al*., (2007) found that the majority of the genetic differentiation of *Ptm* occurred within fields rather than between fields or regions. However, these two studies differ in their degree of differentiation*,* Bogacki *et al* (2010) finding a very low degree of differentiation between *Ptm* field populations in line with the results of this study whereas Serenius *et al*., (2007) finding a much larger degree between states and fields (21.94% and 11.24%, respectively) with low variation within fields (66.82%). In this study, 98% if the genetic variation occurred within fields and only 1.6% occurred between fields and/or regions.

There are different explanations for the contrasting results between our study and previous research; greater distances between sampling sites should mean less migration and therefore larger differences should be found between sampling locations than within the same fields. However, when comparing fields from Agzone 2 and Agzone 6, which are 650 km apart from each other, genetic diversity within fields is significantly higher than between fields. A more likely explanation for the differing results is the molecular marker technologies used to differentiate isolates, with the major factor affecting resolution appearing to be a comparatively low number of genetic markers in the earlier studies.

Genotypic clustering is associated with fungicide resistance

Three of the markers identified by DAPC associated with population differentiation were located on Chromosome 6, within 31 Kbp of the demethylase inhibitor (DMI) fungicide resistance associated gene *Cyp51A* (Mair et al., 2020) and two of these markers contributed most to differentiation. A second group of markers implicated with fungicide resistance were located within 3 kb of a *Ptr* orthologue encoding a benomyl/methotrexate resistance-like protein, and may indicate an alternate mode of fungicide resistance in *Ptm*. Related genes are implicated in multi-drug resistance (MDR) and are part of the Major Facilitator Superfamily (MFS). MDR transporters are proton antiporters that mediate the efflux of a diverse range of drugs and toxic compounds. For example, they have been implicated in providing resistance to quinidine (Vargas et al., 2007), amiloride (Stolz, Wöhrmann, & Vogl, 2005) and fluconazole (Keniya, Fleischer, Klinger, Cannon, & Monk, 2015) in yeast. In the wheat pathogen, *Zymoseptoria tritici*, MDRs provide enhanced fungicide resistance to tolnaftate, terbinafine, the demethylation inhibitor (DMI) metconazole, the quinone outside inhibitor (QoI) azoxystrobin, and the succinate dehydrogenase inhibitor (SDHI) boscalid (Omrane et al., 2017). These marker associations concur with an international study of *Ptt*, where markers nearby to *Ptt Cyp51A* and a MFS domain-containing protein were also implicated in underlying population structure (Dahanayaka et al., 2021).

Other markers that were associated with genetic structuring included a predicted *Ptm* non-ribosomal peptide synthase (NRPS) gene that showed homology to a *Ptr* gene annotated as HC-Toxin. HC-toxins are a form of host selective toxins that are thought to operate through the prevention of defence gene expression. For example, HC-toxin, is a tetrapepetide produced by *Cochliobolus carbonum*, which inhibits histone deacetylase leading to disease symptoms on susceptible plants (Brosch, Ransom, Lechner, Walton, & Loidl, 1995). However, the predicted product of the *Ptm* gene in question is a likely a pentapeptide based on the number of NRPS gene modules, therefore the function may be different. Other cyclic pentapeptide host selective toxins such as victorin, produced by *Cochliobolus victoriae*, are known to exist, however these are not currently considered NRPSs (Kessler et al., 2020). NRPSs also produce other bio-active secondary metabolites; such as those involved in cellular development and stress response (Keller, Turner, & Bennett, 2005). The possible role of this gene in differential virulence of *Ptm* isolates may warrant further examination in future studies.

Additional *Ptm* genes orthologous to annotated *Ptr* genes included: A calcium channel protein 1 (*CCH1*), which may have a role in homeostasis and virulence in *Aspergillus fumigatus (de Castro et al., 2014)*; tRNA A64-2'-O-ribosylphosphate transferase protein, which modifies cytoplasmic initiator tRNAs, preventing them from participating in translational elongation (Kiesewetter, Ott, & Sprinzl, 1990); finally, a pumilio domain containing protein, implicated in post-transcriptional regulation influencing mRNA stability, translation, and localization (Wang, Ogé, Perez-Garcia, Hamama, & Sakr, 2018).

This study has revealed a single Australia-wide population of *Ptm* suggesting unconstrained movement of genotypes within Australia. There also appears to be a lack of recent directional selection although we detected cryptic populations. This is consistent with the most popular barley varieties being susceptible to SFNB (Paynter & Khan, 2024), while the cryptic populations appear to be related to generalised adaptions, in particular the widespread application of fungicides, but may include other environmental or biotic stresses.

Incorporating new resistance genes into modern cultivars is the most cost-effective approach to combatting serious crop diseases such as SFNB. This research is of significance to breeders and growers alike as long-term control measures require an improved understanding of plant-pathogen interactions. *Ptm* as genotypically diverse and mobile pathogen, underlying a need for nationally coordinated disease management and resistance breeding strategies. The challenge to breeders in

developing new varieties will be in extensive testing against the range of known pathotypes and in deploying resistance genes combinations that reduce the likelihood of their breakdown.

Data accessibility

This chapter resulted in a manuscript that was published in the peer reviewed journal, *G3*. This chapter is a reproduction of the manuscript. External tables found in this chapter, including Table S1, Table S2 and Table S3, too large to practically fit in this thesis, is available from https://doi.org/10.25387/g3.21611043.

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GLOBAL SPREAD, DIFFERENTIATION AND SELECTION OF SPOT FORM NET BLOTCH.

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Abstract

Spot form net blotch, caused by *Pyrenophora teres* f. *maculata*, is a significant necrotrophic disease of barley that spread world-wide in the 20th century. Genetic relationships were analysed to determine the genetic diversity, survival and dispersal of a diverse collection of 346 isolates from Australia, Southern Africa, North America, Asia Minor and Europe. The results, based on genome-wide DArTseq data, indicated isolates from Turkey were the most differentiated with regional sub-structuring, together with individuals closely related to geographically distant genotypes. Elsewhere, population subdivision related to country of origin was evident, although low levels of admixturing was found that may represent rare genotypes or migration from unsampled populations. Canadian isolates were the next most diverged and Australian and South African the most closely related. With the exception of Turkish isolates, multiple independent *Cyp51A* mutation events (which confer insensitivity to demethylation inhibitor fungicides) between countries and within regions was evident, with strong selection for a transposable element insertion at the 3' end of the promoter and counter-selection elsewhere. Individuals from Western Australia shared genomic regions and *Cyp51A* haplotypes with South African isolates, suggesting a recent common origin.

Introduction

Spot form net blotch (SFNB) is a major disease of barley, causing yield losses of up to 20% in susceptible cultivars in Australia and quality downgrades associated with increased levels of undersized grain (McLean, Poole, Santa, & Hollaway, 2022). The disease is caused by *Pyrenophora teres* f. *maculata* (*Ptm*), a filamentous ascomycete within the class Dothideomycetes. *Ptm* is closely related, but genetically distinct from, *P. teres* f. *teres* (*Ptt*, Simon R. Ellwood, Syme, Moffat, & Oliver, 2012; Syme et al., 2018), the cause of net form net blotch (NFNB), producing ovoid rather than net-like striated disease symptoms. *Ptm* is classed as a hemi-biotroph, with a short asymptomatic or biotrophic phase followed by necrotrophy (Liu, Ellwood, Oliver, & Friesen, 2011). Although hybridisation may occur between the two pathogens, this is uncommon (Poudel et al., 2017; Poudel et al., 2019) with hybrids showing lower fitness *in planta*, reduced recombination rates and negative epistasis between parental alleles at several loci (Yuzon et al., 2023). Different genetic interactions with barley, governed by numerous major and minor host genes (Clare, Wyatt, Brueggeman, & Friesen, 2020), supports their genetic autonomy.

The first definitive report of SFNB was by Smedegård-Petersen (1971) in Denmark and the disease has since emerged as notable disease on commercial barley across all major growing regions (reviewed in McLean, Howlett, & Hollaway, 2009). The pathogen persists in stubble as pseudothecia containing ascospores, with both ascospores and later asexual conidia from diseased plants dispersed by air turbulence and water splash (Liu et al., 2011). This dual mode of propagation allows both recombination and clonal expansion, rapidly disseminating new alleles, gene combinations or fungicide resistance mutations that provide an adaptive advantage (Mair et al., 2020; Muria-Gonzalez et al., 2023). Hemi-biotrophs and necrotrophs secrete effectors that noticeably induce cell death (Faris & Friesen, 2020). Susceptibility is dominant in the host, with recessive resistance resulting from the loss or mutation of genes encoding proteins targeted by pathogen effectors (Muria-Gonzalez et al., 2023; Peters Haugrud, Zhang, Richards, Friesen, & Faris, 2019). This is known as the inverse gene-for-gene model, in contrast to dominant host resistance against biotrophs that selects for advantageous alleles or mutations in the pathogen population, such as the loss or alteration of the corresponding pathogen effector gene (Lu et al., 2016). Notable exceptions to the conventional gene-for-gene model are the recessive *mlo* and *rbgh2* genes that prime host defences against powdery mildews (C. Ge et al., 2020; X. Ge et al., 2016; Moolhuijzen, Ge, Palmiero, & Ellwood, 2023). Both models occur in net blotch-barley interactions (Liu et al., 2011; Muria-Gonzalez et al., 2023; Williams et al., 1999), with early host detection of the pathogen at the asymptomatic or biotrophic phase likely determining gene-for-gene resistance, followed by the inverse model in the necrotrophic phase.

The strategic development of durable host resistance and integration with other disease control methods requires an understanding of a pathogen's genetic diversity and population structure, as well as the evolutionary potential and overall population dynamics (McDonald & Linde, 2002) These are dependent on understanding a range of different processes including genetic drift, gene flow, selection, population bottlenecks and the contributions of the sexual and asexual stages to maintaining populations. For example, the challenge presented by *Ptt* was illustrated in an international study by Dahanayaka *et al*. (2021), which suggested high levels of migration between countries, but also regional clustering. An Australian study by Linde and Smith (2019) reflected these results, demonstrating a panmictic population structure across the country, likely maintained by extensive gene flow between regions, but also evidence for elevated linkage disequilibrium levels which the authors ascribed to the restricted host diversity of barley monocultures, together with host genotype adaption. Genetic diversity studies of *Ptm* have been made in several countries; Algeria (Ahmed Lhadj et al., 2022), Australia (Bogacki, Keiper, & Oldach, 2010; Hassett et al., 2023; Lehmensiek et al., 2010; McLean et al., 2014; Serenius, Manninen, Wallwork, & Williams, 2007), Canada (Akhavan et al., 2016), Iran (Vasighzadeh et al., 2021), the Republic of South Africa (RSA, Campbell, Lucas, &

Crous, 2002; Lehmensiek et al., 2010), Sardinia (Rau et al., 2003), and Turkey (Oğuz, Ölmez, & Karakaya, 2019). These studies found high levels of genetic diversity and low levels of clonality and, with the exceptions of Iran (Vasighzadeh et al., 2021) and in a comparison between Australia and RSA (Lehmensiek et al., 2010), little evidence for regional genetic differentiation.

The *Ptm* studies above used a variety of genetic marker methods including random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), and simple sequence repeats (SSRs). RAPDs and AFLPs use anonymous markers, meaning they lack genomic context and often have distorted genomic distribution. SSRs are hypervariable, limiting phylogenetic inferences but enabling individuals to be resolved, and typically low numbers are used which also limits genomic representation. DArTseq is a highly parallel genome-wide approach pioneered by Jaccoud et al. (2001), in combination with next-generation sequencing (Sansaloni et al., 2011), and is based on a restriction enzyme complexity reduction step which selects for low copy and gene-rich coding DNA. The DArT system produces both dominant SilicoDArT markers and co-dominant DArTseq SNP markers. Such dense marker sets allow finer resolution of population structure in fewer samples compared to hypervariable SSRs (Jeffries et al., 2016) and greater resolution of admixturing and hybridisation events than previous methods (Melville et al., 2017). In Western Australia, where the most popular recent barley varieties are susceptible to SFNB (Paynter & Khan, 2024; Shackley, Paynter, Bucat, Seymour, & Power, 2021), DArTseq enabled Hassett *et al*. (2023) to infer genotypic clustering unrelated to geographic origin but associated with the *Cyp51A* gene, mutations of which confer resistance to demethylation inhibitor (DMI, or triazole) fungicides (Mair et al., 2020).

Analyses of *Ptm* populations so far have been limited to isolates representing relatively small geographical regions, while a comparative study of geographically diverse populations would provide a valuable perspective on regional relationships. In this study we compared the intercontinental population structure and genetic diversity of *Ptm* isolates from Australia, Canada, Denmark, Hungary, RSA and Turkey. The main objectives were to investigate the genetic relationships between isolates from different countries and their potential for global spread. This was based on Bayesian and multivariate clustering methodologies, which are effective in detecting admixture or migration events, together evolutionary relationships based on genetic distance, molecular variance and population differentiation measures. In Australia, high levels of DMI resistance has recently developed in *Ptm* (Mair et al., 2020). We therefore compared the *Cyp51A* haplotypes and their resistance phenotypes in the collection to determine if resistance arose as independent events.

Materials and methods

Fungal material

Metadata for 346 *Ptm* isolates used in this study is provided in Supplemental Table 1. Live cultures consisted of: Twenty-four Turkish isolates, collected from 16 locations between 2012 and 2015 (Çelik Oğuz, Ölmez, & Karakaya, 2019); Sixteen RSA isolates collected in 2016 from five locations in the Western Cape; Two hundred and eighty-three isolates were collected from four regions in Australia, with the majority from Western Australia (WA, *n* = 224), followed by Victoria (Vic, *n* = 31), South Australia (SA, *n* = 20) and a region straddling the border of Queensland and New South Wales (Qld and NSW, *n* = 8). Pre-existing DArT data for 251 Australian isolates, collected between 2016 and 2020, was published by Hassett *et al*. (2023), these samples are highlighted in Supplemental Table 1. Additional live isolates included in this study was a DMI sensitive control isolate, U7, collected in 2012 (Mair et al., 2020), isolate M3, collected 2009, which possesses virulence against a seedling resistance gene (Muria-Gonzalez et al., 2023), and isolate M4 sampled from the same site. The *Ptm* reference genome assembly isolate SG1 (Syme et al., 2018), collected in 1996, was used as a standard control between DArTseq genotyping runs.

DNA was provided for ten Hungarian isolates originating from four locations. Most were collected from 2017 to 2018, one from 2007 (H-160), and one from 1992 (H-117.1/1). Twenty-four RSA isolates, collected in 2007 from three locations in the Western Cape (Lehmensiek et al., 2010), together with H-1016 from Hungary, were used for PCR amplification and sequencing of the *Cyp51A* gene and promoter only as the DNA quality was not suitable for DArTseq (Supplemental Table 2). *Cyp51A* data for isolates 17FRG089 and 18FRG195 was obtained from Mair *et al*. (2020), which represent promoter haplotypes H3 and H5.

Pre-existing DArTseq genotype data from USQ was included for twelve isolates from Canada, collected by Akhavan *et al*. (2015; 2016) between 2010 and 2011 from the provinces of Alberta, Manitoba, and Saskatchewan. DArTseq data was also provided for the 16 RSA *Ptm* isolates described above, and the two older Hungarian isolates (H-160 and H-117.1/1), Western Australian isolate U7 (Dahanayaka, Vaghefi, Snyman, & Martin, 2021), and a type culture from Denmark, CBS 228.76, collected by V. Smedegaard-Petersen at an unknown date, but presumed to be from the 1970's.

Fungal isolation, DNA extraction and genotyping

Single-spored cultures of WA *Ptm* samples collected in 2021 were made by surface sterilising diseased leaf samples before storage as mycelia agar plugs at -80 °C (Hassett et al., 2023). Genomic DNA was extracted from seven-day-old V8-PDA cultures (150 ml/l V8 juice (Campbell's Soups Australia, Lemnos, VIC, Australia), 10 g/l Difco™ potato dextrose agar (Becton Dickinson, Sparks, MD, USA), 10 g/l agar (Sigma Aldrich, St. Louis, MO, USA) and 3 g/l $CaCO_3$) by scraping mycelia from a single plate. The tissue was freeze-dried in 1.5 ml tubes and DNA extracted using a Wizard Genomic DNA Purification Kit (Promega, Fitchburg, Wisconsin, USA) in accordance with the manufacturer's protocol. DNA concentration and quality were measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific, MA, USA). DNA used for sequencing the *Cyp51A* gene and promoter of isolates from Australia, RSA and Turkey, was extracted by the same method. DNA provided by collaborators for the Hungarian and RSA 2007 isolates was extracted by the cetyl trimethyl ammonium bromide (CTAB) method described by Doyle and Doyle (1987). DArTseq genotyping was performed as described in Hassett *et al*. (2023) by Diversity Arrays Technology Pty Ltd (Canberra, Australia). Genome complexity reduction was achieved with *Pst*I and *Mse*I restriction enzymes, followed by ligation with adapters compatible to the restriction enzyme cleavage sites and containing a sample barcode region before next-generation sequencing on a HiSeq2000 DNA platform (Illumina, USA) Illumina sequences were processed using proprietary DArT analytical pipelines (Sánchez-Sevilla et al., 2015). In a primary

pipeline, FASTQ files are filtered for poor-quality sequences, applying stringent selection criteria to the barcode region of adapters to assign sequences to specific samples. Identical sequences are collapsed into FASTQCOL files, which are then used in a secondary pipeline to call SNP and silicoDArT polymorphisms. The genomic locations of DNA fragments were obtained by BLASTN against a local database containing the *Ptm* isolate SG1 reference genome assembly (GenBank ID: GCA_900231935), with an expected value (E) of < $5e^{-7}$ and a minimum nucleotide identity of 70%.

Integrating DArT data from different genotyping assays and data filtering

Data from different DArTseq genotyping runs was merged in Excel based on AlleleID, which refers to specific genetic markers (both SNP and SilicoDArT) and their alleles. Only markers in common between three independent DArT runs were retained. Data was filtered using *poppr* v 2.8.3 (Kamvar, Tabima, & Grünwald, 2014) within RStudio 4.2.0 (RStudio, Boston, Massachusetts, USA) to remove markers and isolates with >10% missing data using the *missingno* command. Phylogenetically uninformative loci, those containing less than a given percentage of divergent individuals (cut off = 2/n, where n is the number of individuals in a given population) and a minimum allele frequency (MAF) of < 0.01, were also removed using the *informloci* command. The resultant SNP and silicoDArT data are provided in Supplementary Tables 2a and 2b, respectively and at https://doi.org/10.25917/9ezd-6v72.

Identification of multi-locus genotypes

To account for genotyping errors which may result in isolates being placed in different multi-locus genotypes (MLGs), the minimum genetic distance between *Ptm* isolate SG1 replicates in each DArT plate was established. At least one SG1 replicate per plate within each DArTseq run was used and a distance matrix calculated in *poppr* v 2.8.3 (Kamvar et al., 2014) using the *provesti.dist* function. The maximum genetic distance between SG1 replicates provided the minimum cut-off score to classify independent MLGs using the *mlg.filter* command. Clones within the same MLG were removed from subsequent analyses using the *poppr clonecorrect* function.

Genetic diversity, linkage disequilibrium, and AMOVA

Genetic diversity indices for groups of isolates were generated within *poppr* v 2.8.3 (Kamvar et al., 2014) using the *poppr* function. Indices were determined by comparing the number of MLGs to the expected number of MLGs (eMLG) in the original data and calculating Simpson's corrected index ((N/(N - 1)) λ) of multi-locus genotype diversity, Nei's unbiased gene diversity index, and the Shannon–Wiener index of MLG group genotypic diversity. The extent of random mating occurring within populations was determined by calculating gametic equilibrium using the standardised index of association ($\bar{r}_d$), which is independent of sample size (Agapow & Burt, 2001), using 999 permutations. Analysis of molecular variance (AMOVA) differentiation statistics of groups, based on their regional collection sites, were calculated by using *poppr.amova*. Significance was measured using the *randtest* command with 10,000 repetitions. Pairwise PhiPT, to measure of population differentiation, was calculated for clone corrected groups within GenAlEx 6.5 with 999 permutations (Peakall & Smouse, 2006, 2012).

Phylogenetic tree construction

Genetic relationships among clone-corrected isolates were examined using genetic distance data, based on the Prevosti's absolute genetic distance (Prevosti, Ocaña, & Alonso, 1975) and calculated using the *prevosti.dist* command in *poppr* v 2.8.3 (Kamvar et al., 2014). Gene sequence SNPs were coded as a single nucleotide variation and *Cyp51A* promoter transposon insertion variants were coded as presence or absence (1 = transposon variant present, 0 = absent) within Genalex (Peakall and Smouse 2012). Branch support values were calculated with 1000 bootstrap replicates in the *poppr* v 2.8.3 using the *aboot* command. To simplify the dendrogram in Figure 3—1, Australian isolates were reduced in number by random sampling of isolates from the main Australia collection but retaining

the *Ptm* genome reference isolate SG1 and isolates with a HR fungicide phenotype, as STRUCTURE and DAPC results suggested admixturing. Figure 3—1 branch tips were linked to geolocations via the *phylo.to.map* command in *phytools* v 1.9.16 (Revell, 2012).

Genetic clustering and subdivision analyses

Genetic relationships on the entire clone-corrected collection of isolates were compared by principal component analysis (PCA), discriminant analysis of principal components (DAPC), and Bayesian inference using the program STRUCTURE v 2.3.4 (Pritchard, Stephens, & Donnelly, 2000). PCA and DAPC do not make *a priori* assumptions of population structure, with DAPC optimized for large datasets and providing a higher resolution than PCA of between-group variability and population structures among clusters (Jombart, Devillard, & Balloux, 2010). STRUCTURE is a model-based clustering approach that assumes Hardy–Weinberg equilibrium among loci which is able to accommodate admixture linkage disequilibrium to detect population subdivisions (Falush, Stephens, & Pritchard, 2003).

PCA was performed using the *glpca* function in the R package *adegenet* to observe the impact of eigenvalues on the overall variance explained (Jombart, 2008). DAPC was performed using the *find.clusters* command in *adegenet* to produce a K-means clustering graph with the lowest Bayesian information criteria (BIC), then using the appropriate value for each of the DAPC analyses. DAPC analyses were then run using the *dapc* command, based on the cross-validation function *Xval.dapc* result to retain the correct number of PCs (Dray & Dufour, 2007). STRUCTURE was run with a burn-in period of 100,000 steps and 100,000 replications, assessing *K* values between one and nine with 10 iterations. The STUCTURE output data was parsed through STRUCTURE HARVESTER (Earl & vonHoldt, 2011) to determine the optimal *K* value.

Cyp51A promoter and gene DNA sequencing

The *Cyp51A* coding region and 846 bp of the promoter were amplified and direct Sanger sequenced as described in Mair *et al*. (2016). However, two new reverse primers were made to improve sequence quality close the 3' end of *Cyp51A* due to the proximity of mutations. These were (5'- 3'): PtCyp51A_4F GGTATCTCAGCCAACAGCG, nested within the gene, and PtCyp51A_4R GCACTCGTGGTACGTACTGC, which is located after the stop codon. DNA concentrations were measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific, MA, USA) and single amplicons verified by electrophoresis in a 1% agarose gel. PCR products were sequenced by Macrogen (Seoul, South Korea) on an Applied Biosystems ABI3730xl DNA Analyzer 96-capillary array (Thermo Fisher Scientific, Waltham, MA, USA). *Cyp51A* gene and promoter sequences were aligned against the *Ptm* reference isolate SG1 (assembly GCA_900231935, GenBank accession MT499776 and surrounding regions) in Geneious 8.0.1 (Biomatters, Auckland, New Zealand) using the MUSCLE alignment tool, then visually inspected for polymorphisms. DNA sequences are available from GenBank under accessions: OR734722-OR734732, OR751399, OR751400, OR734239-OR734251, and OP753350-OP753352.

In vitro fungicide sensitivity phenotypes

Ptm isolates were tested against six concentrations of tebuconazole; 0 µg ml^{-1}, 2.5 µg ml^{-1}, 5 µg ml^{-1}, 7.5 µg ml^{-1}, 10 µg ml^{-1}, and 20 µg ml^{-1}. Canadian, Hungarian and RSA 2007 isolates were not included as no live cultures were available. Isolates were grown for five days on a V8-PDA plate, then approximately 50 mg of hyphae was scraped from the surface and homogenised in a 1.5 ml Eppendorf tube before adding 200 µl of sterilised deionised water. Ten microliters of inoculum was transferred into 75 µl yeast bacto acetate media (10 g yeast extract, 10 g Bacto peptone, and 10 g sodium acetate per litre, amended to one of the six tebuconazole concentrations above) in 96-well plates (Corning, NY, USA), with three biological replicates per isolate. Isolate growth was visually assessed at three and five days post inoculation, based on the presence or absence of mycelia with the latter verified under a binocular microscope. Isolates showing no hyphal growth were considered sensitive at a given

concentration, those where hyphal growth was evident between 2.5 µg ml^{-1} and 5 µg ml^{-1} were scored as moderately resistant (MR, none of these isolates grew at 7.5 µg ml^{-1}), and isolates with hyphal growth at 10 µg ml^{-1} were scored as highly resistant (HR). No isolates grew at 20 µg ml^{-1}.

Minimum spanning networks based on *Cyp51A* haplotype data

The Excel add-in GenAlEx v 6.5 (Peakall & Smouse, 2006, 2012) was used to manually curate of the location and nucleotide identity of each SNP in the *Cyp51A* gene and promoter region. The data was imported into *poppr* v 2.8.3 (Kamvar et al., 2014) within RStudio 4.2.0 (RStudio, Boston, Massachusetts, USA) using the *getfile* command. Provesti's genetic distance (Prevosti et al., 1975) was used to produce the minimum spanning networks using the *provesti.distance* function. Haplotype networks were constructed using the *plot_poppr_msn* command. Within each node, isolates were grouped to represent the country of origin and fungicide resistance phenotype, while node sizes and their sectors were scaled to indicate the number of isolates from a given group.

Results

Detection of multi-locus genotypes

DArTseq genotyping of *Ptm* samples provided a total of 2,193 SNP and 5,092 SilicoDArT markers shared between independent DArTseq runs (Supplementary Tables 2a and 2b). Of these, 543 SNPs and 690 SilicoDArT markers were retained for analysis after filtering by call-rate and missing data, in total 1,233 markers. The maximum genetic distance between isolate SG1 replicate sample controls was determined to be 0.0085, which was used to contract the number of MLGs. This yielded 338 individual MLGs from 346 *Ptm* isolates: 276 were Australian, 12 Canadian, 15 from RSA, 10 Hungarian, 24 Turkish, and a single MLG from a historical Danish type isolate. No MLGs were shared among countries or between regions within a country, and only four MLGs were composed of more than one isolate. These were predominantly from the more intensively sampled sites in Western Australia, with three groups and ten isolates, and one group of two isolates from Protem in RSA.

Genetic diversity and differentiation between countries

Nei's unbiased gene diversity ranged from 0.13 to 0.25, averaging 0.20 (Table 3-1). Isolates from Turkey had the highest diversity, with Australia and Hungary the lowest. The corrected Simpson's index, which indicates the probability two randomly selected isolates in a population have a different genotype, suggested high MLG diversity for all populations (1-λ > 0.99). This is supported by low standardised index of association ($\bar{r}_d$) values, ranging from 0.010 to 0.043, suggesting predominantly sexual reproduction, with the exception of Turkish isolates with a value of 0.176.

Table 3-1. Genetic diversity indices for groups of *Ptm* isolates from five countries based on DArTseq data. The indices were generated using the *poppr* function within *poppr* v 2.8.3 (Kamvar et al., 2014). Methods for generating genetic diversity indices requires at-least two isolates per group (country). As there is only a single isolate representative from Denmark, no genetic diversity indices can be generated.

	n	**MLG**	**eMLG**	***H***	**1-λ**	H_{exp}	$\bar{r}_d$
Country							
Australia	283	276	9.98	5.6	1.000	0.144	0.010
Canada	12	12	10	2.48	1.000	0.156	0.010
RSA	16	15	9.62	2.69	0.992	0.163	0.043
Hungary	10	10	10	2.3	1	0.131	0.030
Turkey	24	24	10	3.18	1.000	0.248	0.176
Total	346	338*	9.99	5.82	1.000	0.204	0.081

n: Number of isolates in a sample group.
MLG: The total number of unique multi-locus genotypes (MLGs) from each country. *Indicates the cumulative number of MLGs irrespective of field or region.
eMLG: The expected number of MLGs.
H: Shannon-Wiener index of MLG group genotypic diversity, a measure of the number of unique genotypes and their homogeneity.
1- λ: Corrected Simpson's index of MLG diversity, the probability two isolates from the same dataset are different genotypes.
H_{exp}: Nei's unbiased gene diversity index, the probability that two randomly selected alleles are different.
$\bar{r}_d$: The standardised index of association, with a value of zero for a null hypothesis a population is freely recombining.

The higher Turkish value may due to population substructure which increases genome-wide linkage disequilibrium estimates both theoretically (Li & Nei, 1974) and empirically (e.g. Andolfatto &

Przeworski, 2000; Browning & Browning, 2011), influenced by genetic bottlenecks, the mixing of individuals between subpopulations that have different allele frequencies and where selfing or inbreeding is prevalent (Slatkin, 2008; Wright, Lauga, & Charlesworth, 2003).

AMOVA of the entire clone corrected data showed significant genetic differentiation of *Ptm* isolates ($P < 0.01$) both within and among countries, with 53% of the genetic variation occurring among countries, and 47% occurring within (Supplemental Table 3). Differentiation among regions within countries was not significant except in Turkey (67.17%, $p < 0.01$). Pairwise population differentiation as defined by PhiPT was highest between Australian and Canadian populations (0.58), followed by Australian and Turkish populations, and lowest between the Australian and RSA populations (0.34, Supplemental Table 4).

Genetic relationships between *Ptm* isolates based on genetic distance

A phylogenetic dendrogram based on Prevosti's genetic distance model (Prevosti et al., 1975) was produced using a subset of the Australian isolates, as well as all isolates from the other countries (Figure 3—1). The dendrogram showed clustering based on country of origin with a few exceptions that were consistent with PCA, DAPC and STRUCTURE results below. The Turkish isolates from Central Anatolia were the most distant to all other isolates, while isolates from southeastern Turkey group most closely to Australian isolates. The tree also showed that a Turkish isolate 'Edirne' from the Edirne Province, which is on the European side of the Turkish Straights, was most similar to the Hungarian isolates. One Turkish isolate, 13-181, formed an outgroup to branches leading to the Australian, Hungarian and RSA isolates. This contrasted with Gps 276, collected from *H. bulbosum*, which grouped with Turkish *H. vulgare* isolates sharing a branch with Australian isolates, suggesting an overlapping host range. Several Australian isolates shared a common branch the with RSA isolates and isolate M3 was the most diverged, branching outside of the main group and supporting the STRUCTURE cluster membership allocation described below. The single Danish type isolate (CBS 228.76) did not group with Hungarian isolates, lying between these and the Canadian clade.

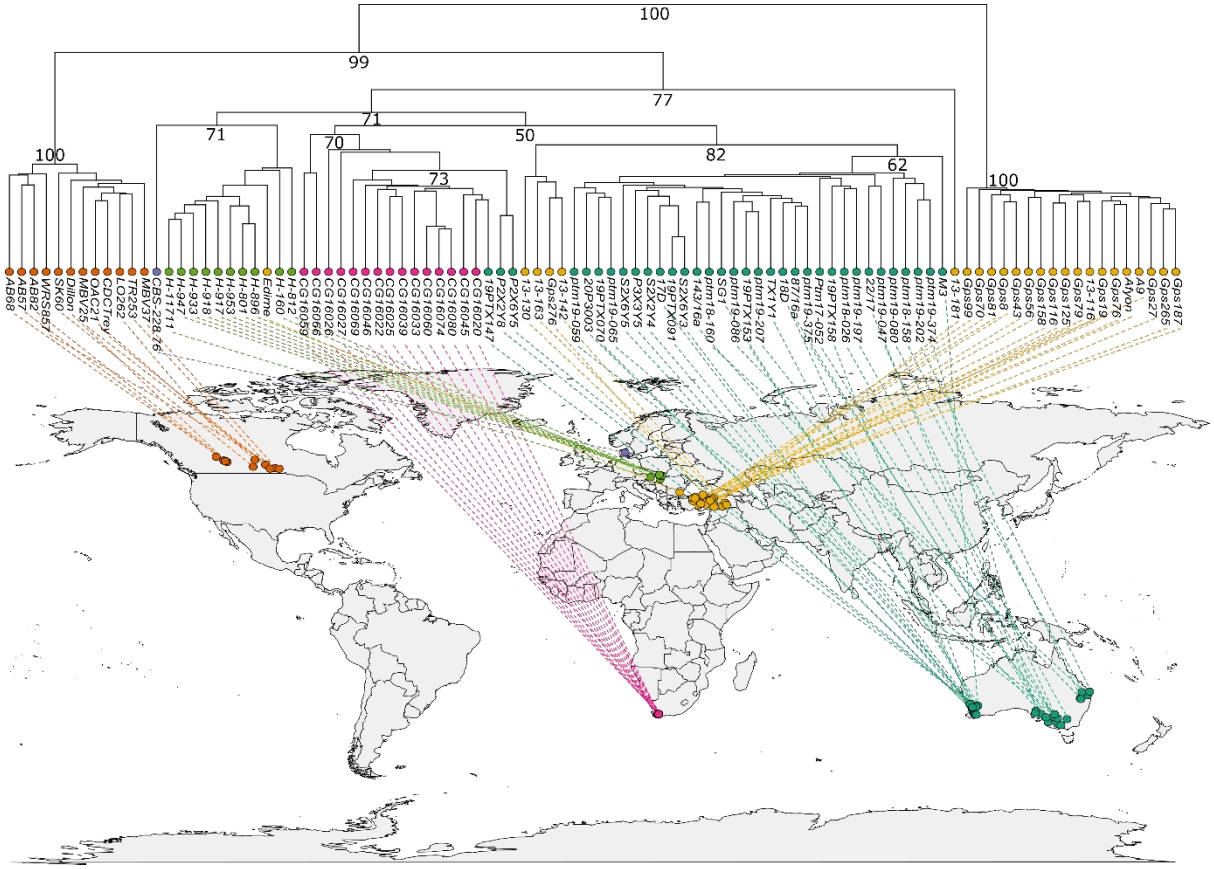

Figure 3—1. Genetic relationships between *Ptm* isolates and their collection locations. The dendrogram is based on Prevosti's distance model (Prevosti et al., 1975) using DArTseq data from isolates from Australia (n = 34, in cyan), Canada (n = 12, tan), Denmark (n = 1, purple), RSA (n = 15, deep pink), Hungary (n = 10, green) and Turkey (n = 24, yellow). Bootstrap support values above 50% are shown at major branch points.

Principal components and multivariate clustering analyses

The genetic relatedness of all 338 MLGs was assessed by principal component analysis (PCA) and discriminant analysis of principal components (DAPC, an unsupervised multivariate clustering method used to assign isolates to groups of related individuals without *a priori* assumptions). Both approaches provided similar results to the distance-based analysis. The first two PCA components explained 17.9% and 6.2% of the variation (Supplemental Figure 9) and assigned most isolates to their countries of origin, although a proportion occupied intermediate positions between 95% confidence ellipses (n > 25). DAPC suggested models of between four and six clusters (Supplemental Figure 10), with $K = 5$ the optimal value for subsequent analysis. More isolates were assigned to their host countries than PCA, particularly for isolates from Hungary, Canada and Turkey (Supplemental Figure 11). However, six Turkish isolates and the Danish type isolate noticeably grouped with the Hungarian isolates, indicating the existence of related alleles (Jombart et al., 2010), and one RSA isolate grouped with the Canadian cluster. The majority of the Australian isolates formed a single cluster, and 11 isolates from Western Australia grouped with RSA isolates, as opposed to only two assigned by PCA. These were the same isolates identified as an outgroup in a previous study by Hassett *et al*. (2023).

Model-based clustering analysis

The Bayesian-inference program STRUCTURE was also used to assign genetic groupings, based on an optimal number of two clusters determined by STRUCTURE HARVESTER (Supplemental Figure 12). At

this level of differentiation, the Australian isolates belonged to a single cluster and most isolates from Turkey composed a second cluster (Figure 3—2). Isolates from RSA, Hungary and Canada showed mixed origin. Using a 70% cut-off score to assign isolates to a single cluster, all Australian, most RSA (n = 14), most Hungarian (n = 6) and the majority of Turkish isolates from the southeast of the country (n = 5) grouped into Cluster I, while only Turkish isolates from Ankara, the Black Sea and Eastern regions formed Cluster II (collectively termed Central Anatolian isolates). All Canadian, a single RSA, four Hungarian, the single Danish isolate and a Turkish isolate showed intermediate membership of both clusters.

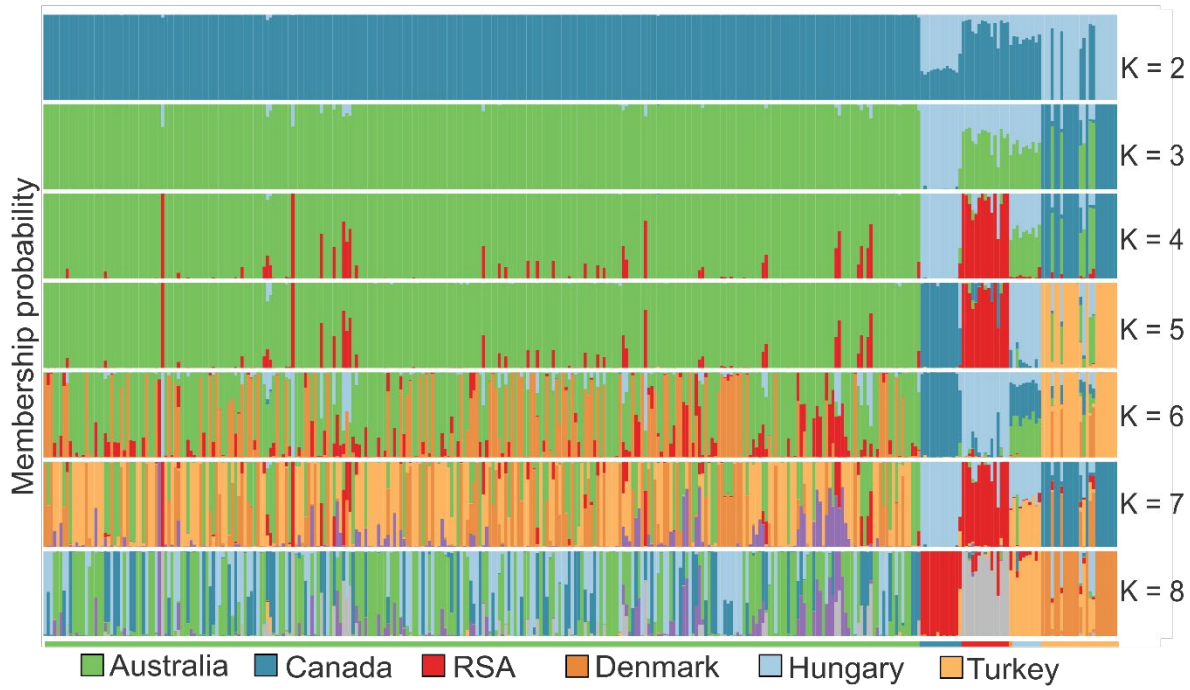

Figure 3—2. Estimates of genetic structuring in the entire clone-corrected *Ptm* collection grouped into clusters (K = 2-8) in STRUCTURE. The coloured bar at the base of the figure represents isolates grouped by country; Australia (n = 276), Canada (n = 12), RSA (n = 15), Denmark (n = 1), Hungary (n = 10) and Turkey (n = 24), respectively. Vertical bars represent individual isolates, and the colour proportions in each bar indicates the estimated membership fraction of each individual to each cluster.

In common with the PCA and DAPC results, population structure by country was visible in the STRUCTURE results up to *K* = 5. Australian isolates appeared to be composed of mixed origin with a larger primary cluster and a smaller admixed group. Isolates with membership to the admixed group (n = 8, cut-off >50%) were made up of a similar sub-group identified in the DAPC results as clustering closer to the RSA population. Additional distinctive isolates found only in Western Australia included M3 and M4, collected from the same location in 2009, which appear to share modest and different membership proportions (cut-off = >5%) with Hungary and RSA, despite belonging to the main Australian population.

Fungicide resistance status of Australian, RSA and Turkish isolates

Tebuconazole, a widely used representative of the DMI group of fungicides, was used in discriminatory dose screens to compare resistance phenotypes. The tests were performed on a selection of West Australian isolates that clustered with the RSA isolates, referred to as RSA-like, contained within the DAPC subset and at *K* = 4 in STRUCTURE with a RSA similarity Q-score of > 0.20 (n = 14), the 2016 RSA isolates (n = 14), randomly selected isolates from the primary Australian DAPC cluster (n = 29), and a selection of Turkish isolates (n = 4). Additional screens showed the Turkish isolates were sensitive to tebuconazole (data not shown) and four were selected from groupings based on genetic distance (Figure 3—1). Two (Gps43 and Gps76) were from Central Anatolia, representing the group of isolates most distant to all other isolates. The remaining two were from Southeastern Anatolia, with one (13-163) belonging to a clade next to the Australian isolates and the second (13-181) representing an outgroup to isolates from Australia, RSA and Europe. All the RSA-like isolates were highly resistant (HR) with the exception of isolate M3. Approximately half of the Australian primary cluster isolates were moderately resistant (MR, n = 14), and the remainder were sensitive (n = 15). Most 2016 RSA isolates (n = 12) were HR with two MR, while the Turkish isolates were sensitive (Supplemental Table 5).

Ptm Cyp51A gene sequence comparisons

Mair *et al.* (2020) previously studied *Ptm* fungicide sensitivity and *Cyp51A* gene sequences in WA and described five *Cyp51A* SNP-based haplotypes (H1 to H5). DMI sensitive isolates were present in H1 and H2, with H2 also containing MR1 isolates, defined as containing a LTR retrotransposon-like promoter insertion element and being moderately resistant to tebuconazole and resistant to epoxiconazole. HR isolates were found in H3 and H4 haplotypes, while H5 contained MR2 isolates, defined as being moderately resistant to tebuconazole but sensitive to epoxiconazole. DMI resistance in H3, H4 and H5 was due to three different F489L mutations: c1467a, c1467g and t1465c, respectively.

To investigate *Cyp51A* variants, the gene and 846 bp upstream of the start codon was sequenced for 95 isolates. These included all the isolates used in the tebuconazole screens described above as well as 24 RSA isolates collected in 2007 and eight Hungarian isolates. Single representatives of haplotypes H3 and H5 from Mair *et al*. (2020) were also included as they were not detected among our samples.

Cyp51A had 26 SNPs across the collection. Excluding F489L mutations, eight were non-synonymous substitutions. Of these, six were exclusive to the Turkish isolates and two to the Australian isolates. Three codon variants led to the non-synonymous F489L mutation and, consistent with Mair *et al*. (2020), all HR isolates contained both the mutation and a *Ty1-Copia* LTR retrotransposon-like insertion in the promoter, whereas all sensitive isolates had the wild-type codon. Moderately resistant isolates also either possessed the F489L mutation or contained a transposon insertion. The majority of Hungarian isolates (n = 7) and all the 2007 RSA isolates contained the F489L mutation (data summarised in Supplemental Table 5).

H1 and H2 haplotypes were found only in Australian isolates. H1 was represented by 27 isolates but H2 only by a single isolate, M3. This isolate was collected from the same location as the single specimen identified in Mair *et al.* (2020), suggesting a local variant. The second most common Australian haplotype was H4 with 13 isolates. Thirteen additional *Ptm Cyp51A* haplotypes to those in the Mair *et al*. (2020) study were found and numbered consecutively (H6-H18). These are defined in Supplemental Table 6, with alignments provided in Supplemental File 1. The sequences are available in GenBank under accessions OR734722-OR734732 and OR751399-OR751400. Most were composed of single isolates including haplotype H6 with Australian isolate S2X2Y4, H7 with Hungarian isolate H801, and H8 with Turkish isolate 13-181. H11 and H17 contained the 2007 RSA isolates PTM55 and PTM57, respectively. H14 contained Hungarian isolate H812, while H15, H16 and H18 contained Turkish isolates GPS76, 13-163, and GPS43, respectively. More common haplotypes included H9, with

isolates from both the 2007 RSA (n = 19) and 2016 RSA (n = 8) collections. H10 included an isolate from Australia, P3X2Y9, and the two MR isolates from the RSA 2016 collection. H12 contained the majority of the Hungarian isolates (n = 6). H13 comprised isolates from both the 2007 (n = 2) and the 2016 RSA collections (n = 3).

A minimum spanning network of the *Cyp51A* gene was produced to illustrate haplotype relationships and their fungicide resistance status (Figure 3—3). Close relationships were apparent between Australian and RSA isolates: haplotype F489L H4 which included one putatively HR isolate from the 2007 RSA collection (PTM24), one HR isolate from the 2016 RSA collection (CG16045), and all 13 HR RSA-like Australian isolates; haplotype H10 which contained two MR RSA isolates and one Australian sensitive isolate; Australian HR haplotype H3 and H17 from RSA (untested) were also closely related. A number of RSA isolates from 2007 (n = 3) and 2016 (n = 2) in H13 showed a strong relationship with the majority of the Hungarian isolates (n = 6) in H7 and H12, reflecting the DArTseq-based PCA and genetic distance-based proximities.

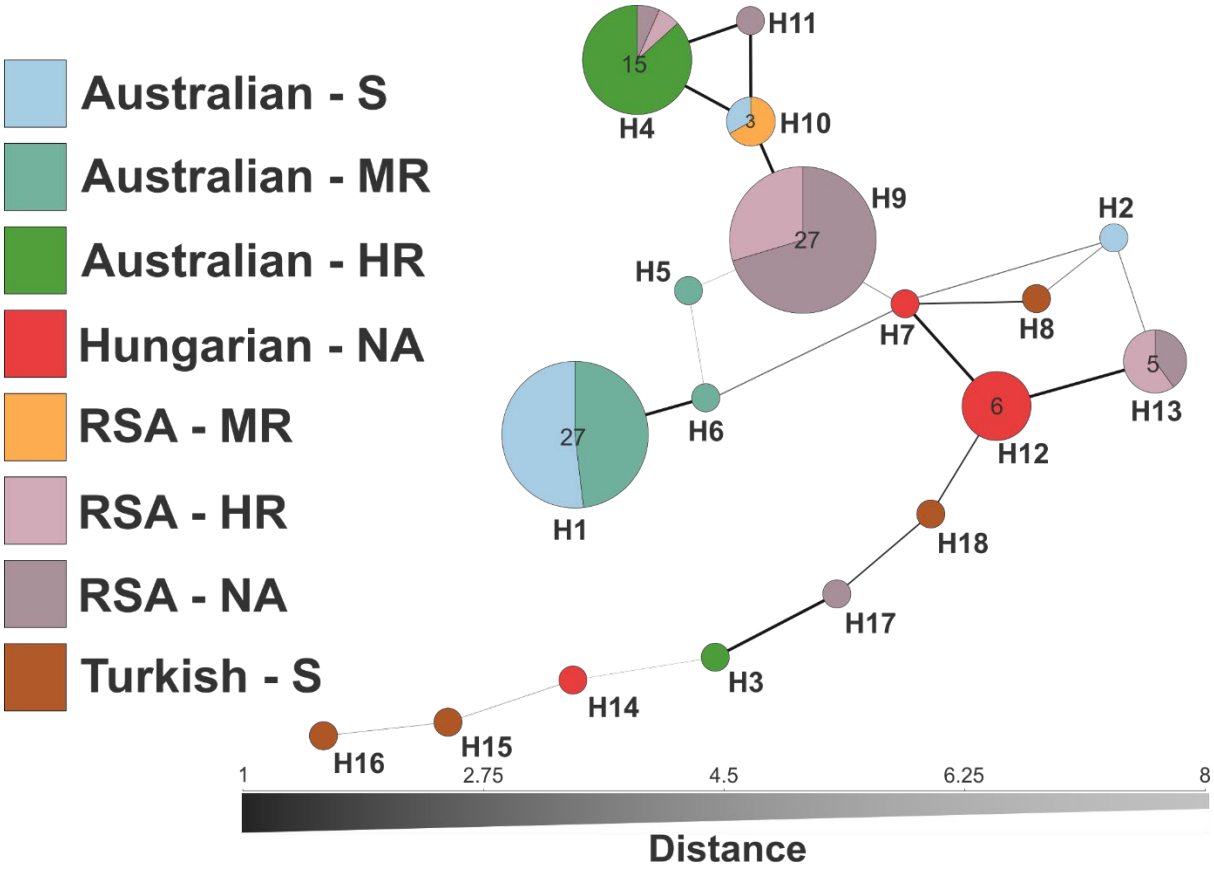

Figure 3—3. Minimum spanning network based on *Ptm Cyp51A* gene sequences comparing international haplotype relationships. Each circle (node) represents a unique haplotype of *Cyp51A* and each node size is proportional to the number of sampled isolates. Isolate origins and fungicide phenotypes are presented as proportionate sectors of nodes with wider and darker lines between nodes representing higher genetic relatedness. HR, MR, S and NA indicate highly resistant, moderately resistant, sensitive isolate responses to tebuconazole or data not available, respectively. Single representative sequences for H3 and H5 were obtained from Mair *et al*. (2020).

Ptm Cyp51A promoter region polymorphisms

Sequence data of the promoter region of *Cyp51A* revealed 32 SNPs and six haplotypes. P1 and P2 were reported by Mair *et al*. (2020) and four new, predominantly Turkish haplotypes, P3 – P6, are novel to this study. A minimum spanning network (Supplemental Figure 13), excluding transposon insertions (see below), shows all tebuconazole HR *Cyp51A* haplotypes were found within node P2, MR isolates were located within P1 and P2 and sensitive isolates were found in all six nodes. As with the *Cyp51A* gene haplotypes, the Turkish sequences are the most diverged. An alignment of P1-P6 is provided in Supplemental File 2 and P3 – P6 sequences are available in GenBank under accessions: OR734240, OR734248, OR734250, and OR734251.

Five different LTR transposon variants were found within the upstream region of *Cyp51A* in the P1 and P2 haplotypes, defined by different combinations of three SNPs. Twelve independent insertion events in the two haplotypes were observed, with insertion of different alleles at two positions giving a total of 14 different transposon variant and location combinations (Supplemental File 3). We propose a new naming convention to account for different transposon alleles at the same position which is presented together with GenBank accession numbers in Supplemental Table 7. In essence the names are based on the two backbone haplotypes on which all mutations conferring resistance are derived (P1 and P2), followed by the promoter transposon insertion position relative to the *Cyp51A* start codon, then the *Ty1*-Copia LTR retrotransposon-like insertion allele number. The more notable of these include: P2:90:1 and P2:95:2, which are present in HR isolates from Australia and the RSA and with one RSA isolate, PTM24, possessing the same *Cyp51A* F489L codon found in Australia; P2:46:5, which is shared between Australian and Hungarian isolates; while MR isolates from Australia have unique transposon insertion haplotypes (Supplemental Table 5).

A dendrogram combining all *Cyp51A* gene and promoter sequence data together with promoter transposon insertion sites and *Cyp51A* 489 codon usage was produced to summarise and distinguish the inter-relationships of variants (Figure 3—4). The figure illustrates the range of unique transposon insertion sites in combination with F489L mutations present in *Ptm* and highlights the key divergence point between the P1 and P2 base promoter haplotypes, which separates P2 predominantly HR isolates (containing both F489L mutations and transposon insertions) from P1 MR isolates (containing transposon insertions only) and S isolates. The RSA isolates possessed the largest number of P2 transposon insertion alleles (seven), which may indicate selection over a longer period of time. This compared with three in Australian P2 isolates, followed by four for Australian P1 isolates. The only completely identical SNP and insertion profile (H4-P2:90:1) found between countries is boxed in blue and depicts the 2007 RSA isolate PTM24 and the majority of the Australian HR isolates.

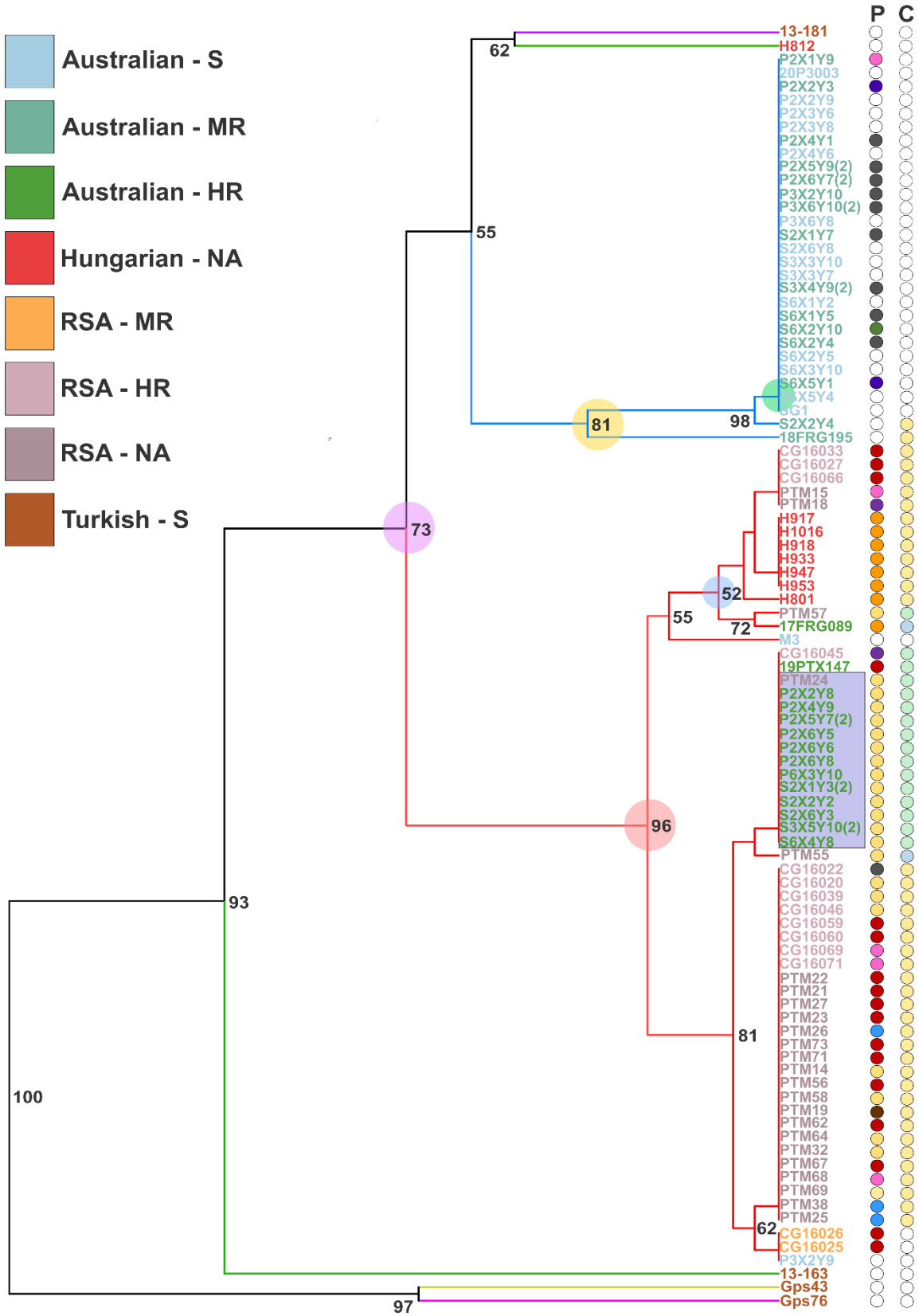
Australian - S
Australian - MR
Australian - HR
Hungarian - NA
RSA - MR
RSA - HR
RSA - NA
Turkish - S
P
C
13-181
H812
P2X1Y9
20P3003
P2X2Y3
P2X2Y9
P2X3Y6
P2X3Y8
P2X4Y1
P2X4Y6
P2X5Y9(2)
P2X6Y7(2)
P3X2Y10
P3X6Y10(2)
P3X6Y8
S2X1Y7
S2X6Y8
S3X3Y10
S3X3Y7
S3X4Y9(2)
S6X1Y2
S6X1Y5
S6X2Y10
S6X2Y4
S6X2Y5
S6X3Y10
S6X5Y1
S6X5Y4
CG1
S2X2Y4
18FRG195
CG16033
CG16027
CG16066
PTM15
PTM18
H917
H1016
H918
H933
H947
H953
H801
PTM57
17FRG089
M3
CG16045
19PTX147
PTM24
P2X2Y8
P2X4Y9
P2X5Y7(2)
P2X6Y5
P2X6Y6
P2X6Y8
P6X3Y10
S2X1Y3(2)
S2X2Y2
S2X6Y3
S3X5Y10(2)
S6X4Y8
PTM55
CG16022
CG16020
CG16039
CG16046
CG16059
CG16060
CG16069
CG16071
PTM22
PTM21
PTM27
PTM23
PTM26
PTM73
PTM71
PTM14
PTM56
PTM58
PTM19
PTM62
PTM64
PTM32
PTM67
PTM68
PTM69
PTM38
PTM25
CG16026
CG16025
P3X2Y9
13-163
Gps43
Gps76
62
55
81
98
73
52
55
72
96
93
81
100
62
97

Figure 3—4. Phylogram of *Ptm* relationships based on *Cyp51A* promoter and gene sequences and transposon insertion data. The dendrogram is based on 71 possible differences (58 SNPs and 14 different transposon allele insertion position combinations). Coloured branches indicate divergence events leading to six basal promoter variants in Supplemental Figure 13. The node depicted in purple shows the key branch point separating P1 from P2 isolates (depicted by the yellow and red nodes, respectively). The blue node highlights a transposon insertion found in the Hungarian isolates and the HR Australian 17FRG089 H3-P2:46:5 haplotype. The green node depicts the point past which two new transposon insertions occur in the P1 haplotype of the Australian population (indicated by the dark green and dark blue circles in the promoter column). The boxed isolates contain identical haplotypes from Western Australia and RSA. Full haplotype pedigrees are provided in Supplemental Table 5. P indicates promoter transposon insertion position: white, no insertion; orange, -46, brown; -57; light blue, -62; pink, -66; grey, -74; dark blue, -75; green, -77; yellow, -90; purple, -94 and red, -95. C represents the *Cyp51A* 489 codon nucleotide sequence: white, TTC (wild type); yellow CTC; green TTG and blue TTA. HR, MR, S and NA indicate highly resistant, moderately resistant, and sensitive isolate responses to tebuconazole or data not available, respectively. Bootstrap support values above 50% are shown for major branch points.

Discussion

Human trading and cultivation activities have profoundly impacted the distribution and dispersal of plant pathogens, a process that has accelerated in the current era of globalised transport (Bebber, Holmes, & Gurr, 2014; Sotiropoulos et al., 2022). In this study we examined genetic relationships between *Ptm* isolates in the most geographically widespread collection assembled to date. Pre-existing genotyping data for Western Australian isolates formed the majority of the collection and, based on Nei's unbiased gene diversity index, served as a control to show deeper sampling does not necessarily improve the detection of general regional diversity, however, a few unusual localised patterns were revealed. Overall, the results revealed population subdivision related to country of origin and, with the exception of Turkey, similar amounts of diversity within countries as among countries. Regional selection of *Ptm* was evident, particularly of fungicide resistant isolates, and evidence for admixturing of isolates between RSA and Australia. These results contrast with *Ptt*, which shows greater admixturing of genotypes between countries and deep phylogenetic lineages at the whole genome level (Dahanayaka, Vaghefi, Knight, et al., 2021; Moolhuijzen et al., 2020), perhaps reflecting a longer association with barley (Simon R. Ellwood et al., 2012).

Intercontinental *Ptm* population subdivision

Principal components, genetic distance and STRUCTURE-based analyses indicated that Turkish isolates from Central Anatolia showed distinct separation from all other isolates, while Canadian isolates were also placed in a discrete cluster. Isolates from Australia and RSA were closely related, however, all the analyses supported *Ptm* population subdivision related to country of origin. The greater differentiation of Canadian isolates may be due to adaptation to a distinctive sub-group of barley genotypes (Hill et al., 2021), or a bottleneck effect and/or genetic drift during initial establishment. Australia and the Western Cape of RSA share similar environments and climates which may have driven the selection of common genotypes, as well as a history of shared cultivars such as Clipper (Campbell & Crous, 2002).

The methods above also indicated several isolates with uncertain ancestry, either falling between principal component 95% confidence ellipses, or occupying unexpected positions in the distance-based tree and evidence for admixed individuals in STRUCTURE. DAPC, a multivariate approach that quantifies the contribution of individual alleles to population structuring (Jombart et al., 2010) provided clearer allocation of these individuals to genetic groupings, placing 11/276 of the Australian isolates with the RSA group, one RSA isolate with the Canadian group, and six Turkish isolates and the single Danish isolate into the Hungarian group. However, the DAPC results also indicated a few isolates with ambiguous placements that may represent recent migration events, membership of unsampled populations or uncommon genotypes. For example, two of the six Turkish isolates lay outside of the Hungarian group's 95% confidence ellipse as well as an RSA isolate placed with Canadian isolates.

A Turkish isolate from Edirne was closely related to the Hungarian isolates, a result confirmed by sequence data for a unique effector gene haplotype in Europe (unpublished data). This may be explained by the sampling location, as Edirne lies on the western side of the Turkish Straights, a body of water separating Eastern Thrace, a province of Turkey in southern Europe, and Anatolia. Such a physical barrier may limit dispersion and the exchange of genetic material. The grouping of this isolate, the Danish isolate and those from Hungary suggests modest genetic variation across Europe, although the sample size was low.

High genetic diversity and population sub-structure in Turkey

Turkish *Ptm* isolates from the Central Anatolian plateau showed greater genetic distances from all other isolates. Regional genotypic clustering was observed, a phenomenon not present among isolates from the other countries in this study, with Central Anatolian isolates grouping separately from Southeastern Anatolian and the Edirne isolate which are more closely related to the globally distributed populations. This structuring may reflect host selection on the diverse barley genotypes found in Turkey within different environmental niches, as Turkey lies along the northern extent of the Fertile Crescent (Breasted, 1914), which is believed to be the first region where barley was domesticated (Zohary & Hopf, 2000) and a major centre of diversity (Dai et al., 2012; Orabi, Backes, Wolday, Yahyaoui, & Jahoor, 2007). There are significant climatic differences and changes in elevation between Turkish regions, with Southeastern Anatolia having some of the hottest summers, compared to the coolest found in the western Marmara (Sensoy, 2004). Aside from wild barley species such as *Hordeum spontaneum* and *Hordeum bulbosum* (Karakaya, Çelik Oğuz, & Saraç Sivrikaya, 2020; Karakaya, Mert, Çelik Oğuz, Ertaş, & Karagöz, 2016), winter sown feed barley cultivars are common in Turkey (Sipahi, Akar, Yıldız, & Sayim, 2010), and landraces with purple or black grain are grown extensively, particularly in Southeastern Turkey (Ozberk, Ozberk, Ayhan, Bayhan, & Ipeksever, 2020). The southeastern isolates, or other unsampled populations along the Fertile Crescent, might be regarded as the source of closely related genotypes in Australia and elsewhere, or alternatively represent migration to Turkey via introduced modern cultivars.

Çelik Oğuz *et al*. (2019) previously assessed genetic similarities of 49 Turkish *Ptm* isolates and found two main groups but no link between sampling year, mating type or geographical area. In neighbouring Iran, Vasighzadeh *et al*. (2021) found strong population structure among 116 isolates with most of the genetic variation contained within regional populations. Those studies used different types of genetic markers and different PCR product resolution methods (anonymous inter-SSRs resolved by agarose gel electrophoresis and SSRs separated on a sequencing platform, respectively). SSR markers are more similar to DArT markers in terms of detecting specific loci and higher numbers of sequence polymorphisms per individual, which limits comparisons with inter-SSRs although, complimentary to this study, Çelik Oğuz *et al*. (2019) found isolates from Central Anatolia formed the majority of one of their groups.

Fungicide resistant Western Australian *Ptm* isolates share genetic similarities with RSA isolates

Hassett *et al*. (2023) identified a 'cryptic' group of Western Australian isolates by DAPC, with no obvious association with geographic distance or host cultivar. Examination of genes contributing to differentiation implicated the C14α-demethylase (*Cyp51A*) gene, mutations of which underlies resistance to the DMI Group 3 triazole fungicides. DMI fungicides act through C14α-demethylase to inhibit ergosterol biosynthesis, an essential fungal plasma membrane component (Aoyama et al., 1996; Parks & Casey, 1995; Yoshida, 1993) and mutation of phenylalanine (F) to Leucine (L) at codon 489 (F489L) is the primary mechanism of resistance in *P. teres* (Mair et al., 2016; Mair et al., 2020).

In this study, admixture between the same Western Australian isolates and RSA isolates was indicated by DAPC, with support by STRUCTURE population membership assignments at $K = 4$. The STRUCTURE assignments indicated these isolates possess varying proportions of the donor genome. Ellwood *et al*. (2019) found rapid recombination occurred around a new *Ptt Cyp51A* resistance mutation in WA individuals discovered in the wider population some two years after initial reports, indicating the potential for rapid assimilation that may obscure the phylogenetic origin. To further explore their association, the RSA-like Western Australian and RSA isolates were studied by sequencing their

Cyp51A gene and in experiments measuring resistance to the DMI tebuconazole. The RSA-like Australian isolates were HR, while the RSA isolates were MR to HR.

Mair *et al*. (2020) found that a combination of the *Ptm Cyp51A* F489L mutation together with a *Ty1-Copia* LTR retrotransposon-like insertion in the promoter correlated with constitutive *Cyp51A* overexpression to give a HR phenotype to different DMIs. When either the insertion element or the F489L mutation was absent, a MR phenotype was observed and genotypes with neither mutation had a sensitive phenotype. In this study, no new non-synonymous F489L mutations were discovered among RSA and Australian isolates and the same relationship between F489L, transposon insertion site and phenotype were observed. However, new transposon alleles distinguished by SNP changes and their insertion sites were identified in both the Australian and RSA populations, with an identical variant (H4-P2:90:1) shared between them. Furthermore, clustering of the promoter insertions in an approximately 50 bp region close to the start codon was evident, which may indicate disruption of a transcription repressor site, with counter-selection across the remaining 5' region underlining the importance of *Cyp51A* as an essential sterol synthesis gene.

Compromised *Ptm* sensitivity to a range of DMIs was reported in RSA isolates from the Western Cape collected between 1996 and 1997, including against tebuconazole (Campbell & Crous, 2002). The authors commented that selection for fungicide resistance was likely promoted by extensive cultivation at the time of Clipper, a spot form net blotch susceptible cultivar. Most of the isolates appear to have been either S or MR based on the fungicide concentrations tested, but at least one isolate possessed an IC_{50} (half maximal inhibitory concentration) of >20 µg ml^{-1} tebuconazole, suggesting HR isolates have existed since 1997. Genetic similarities of the RSA-like Western Australian isolates together with high levels of pre-existing tebuconazole resistance in RSA isolates indicates either similar ancestry or an event enabling inter-crossing between the two populations. Convergent evolution leading to these isolates may be also be an explanation. However, the existence of the RSA-like WA sub-group isolates only in recent collections and their different RSA-like membership proportions detected in STRUCTURE would argue against this possibility.

Multiple independent DMI fungicide resistance events

Hungarian *Ptm Cyp51A* haplotypes grouped together in a single phylogenetic cluster, and the majority contained non-synonymous F489L mutations and an transposon insertion in the promoter, indicating high levels of tebuconazole resistance which are known to exist in recent European isolates (Lammari, Rehfus, Stammler, & Benslimane, 2020). However, promoter insertions for all the Hungarian isolates were at -46 bp before the start codon, a site not found in the RSA isolates but indicating mechanistic convergent evolution for enhanced DMI resistance.

The only known instance of the -46 bp transposon outside of Hungary was found by Mair *et al*. (2020) in the HR Western Australian haplotype H3-P2:46:5. Nevertheless, there were differences in SNP composition within the *Cyp51A* gene and in the F489L codon (t1465c for Hungarian isolates and c1467a for H3 isolates). No H3 genotypes were detected in this study but it is worth noting they were not found in Australian isolates prior to 2017, with almost all collected from heavily infected cv Oxford plants in south western WA (Mair et al., 2020), suggesting an association with cultivar-specific enhanced virulence, with cv Oxford no longer recommended in sowing guides. The RSA-like HR isolates by contrast were almost entirely sampled north of Perth, confirming independent regional emergence of fungicide resistance.

Two groups of RSA isolates were used in this study, recent (post-2016) and 2007. We were unable to measure resistance in the 2007 isolates as only their DNA was available, but isolates with matching gene and promoter mutations among the 2016 isolates were screened and indicated the older

haplotypes were likely HR to DMI fungicides. Several haplotypes from the 2007 RSA population were absent in the more modern population but they too contained a promoter insertion and F489L mutation, suggesting they were also HR. Notably, H4 isolates, which included representatives of the RSA-like Australian isolates, a 2007 RSA isolate and a 2016 RSA isolate all had identical *Cyp51A* gene sequences, further indicating the similarity of isolates from these regions.

The appearance of different *Cyp51A* F489L mutations and promoter transposon insertions in different *Ptm* populations is an example of convergent evolution. However, there is also evidence of 'soft selective sweeps', a phenomenon whereby multiple alleles at the same locus, present either in standing genetic variation or generated during selection, become prevalent (Delmas et al., 2017; Hermisson & Pennings, 2005). The RSA 2007 isolates possessed three separate F489L mutation alleles, suggesting at least three independent gains of DMI resistance, while multiple examples of different transposon insertions are evident within the WA and RSA populations (Supplemental File 3). The global appearance of these different mutations illustrates how *Ptm* readily generates DMI resistance, seemingly without relying on target site duplications as found in *Ptt*, for which promoter insertions have not been reported (Mair et al., 2016; Turo et al., 2021).

This study provides a baseline of current *Ptm* genotypic diversity between countries and suggests the pathogen has a defined population structure at the international level that is consistent with a model of recent introductions involving genetic bottlenecks and drift with adaption to new environments. Turkish isolates were the most genetically diverged, with regional sub-structuring and with some individuals genetically similar to isolates from other countries. The Turkish isolates also showed the largest number of SNPs in the *Cyp51A* gene and phylogenetically contain the most diverged individuals compared to all other samples in this study. However, they were sensitive when challenged with tebuconazole, which accords with traditional cultivation practices without fungicides. The Australian population appeared autonomous except for a subgroup with genetic similarities to RSA isolates with which they shared similar *Cyp51*A gene and promoter region changes conferring DMI fungicide resistance, suggesting a shared origin of resistance. The range of *Ptm* genotypes uncovered in this study will aid future research investigating host-pathogen interactions for this significant crop disease.

In this study, 346 *Ptm* isolates were characterised from Australia, Canada, Hungary, Republic of South Africa, Turkey, and Denmark. The results, based on genome wide DArTseq data, indicated isolates from Turkey were the most differentiated and showed regional sub-structuring. Globally, population subdivision related to country of origin was evident, however Australian and South African populations were considered the most closely related, with the exception of a few Turkish isolates. Genomic regions associated with fungicide resistance established in Chapter 2 were analysed and found to be similar in individuals from Western Australia and South African isolates, suggesting a recent common origin.

Data accessibility

This chapter resulted in a manuscript that had been accepted for publication at the time of submitting this thesis in the peer reviewed journal, *Phytopathology*. This chapter is a reproduction of the manuscript. External tables found in this chapter, too large to appropriately fit in this thesis, including DArTSeq sequencing data generated from this study is available from https://doi.org/10.25917/9ezd-6v72.

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POPULATION STRUCTURE AND GENETIC RELATIONSHIP OF *PYRENOPHORA TERES* OF BARLEY (*HORDEUM VULGARE*) AND BARLEY GRASS (*H. MURINUM* SSP. *LEPORINUM*)

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Abstract:

The expansion and proliferation of pathogen populations often relies on natural and artificial stepping stones. One such potential stepping stone is the common weed, barley grass (*H. murinum* ssp. *leporinum*), which is phylogenetically related to barley (*Hordeum vulgare*). *Pyrenophora teres* diseases are categorised by their symptomatic forms as either spot form or net form net blotch, with the causal agents in barley being *Pyrenophora teres* f. *maculata* (*Ptm*) or *Pyrenophora teres* f. *teres* (*Ptt*). Both spot and net form symptoms have also been observed in barley grass. Using DArTSeq SNP markers and pathogenicity tests, the *in vivo* host range of the diseases were tested in both barley grass and barley in order to determine if barley grass *P. teres* isolates are a current threat to barley, or if either *Ptm* or *Ptt* have the potential to reside in barley grass found next to fields. Results of this study determined that the spot and net symptoms observed in barley grass are likely unrelated to a separation in subspecies, in contrast to *Ptm* and *Ptt* in barley, and net and spot form isolates on barley grass likely represent a single population.

Introduction

The intricate interactions between plant pathogens and their hosts directly affects the severity and occurrence of diseases. These interactions are greatly influenced by the population size and genetic diversity of hosts and pathogens. More crucially, disease incidence is often interrelated with pathogen transmissibility and host genetic variability. Pathogen genetic diversity contributes to variations in disease outcomes, with genetically diverse pathogens often displaying a higher range of virulence. Host population size also impacts pathogen diversity, as larger host populations tend to favour greater neutral diversity in the pathogen (McDonald & Linde, 2002). Furthermore, pathogens capable of infecting multiple host species often exhibit higher diversity compared to those confined to a single host species (Thrall et al., 2012). Therefore, an understanding of pathogen genetic and pathogenic diversity and the factors driving the evolution of plant pathogens is essential for the development of effective, long-term management solutions of these pathogens on crops and in predicting the emergence of economically relevant disease variants.

In the case of the *Pyrenophora teres*-barley interaction, the role of wild grasses as alternative hosts for *P. teres* and their potential as an inoculum source has been receiving increasing attention in recent years (Çelik Oğuz & Karakaya, 2021; Dahanayaka et al., 2021; Linde & Smith, 2019; Vasighzadeh, Sharifnabi, Javan-Nikkhah, & Stukenbrock, 2022). Concerns have been raised over wild grasses, including barley grass (*Hordeum murinum* L.), wild barley (*Hordeum spontaneum* (K. Koch)), and bulbous barley (*Hordeum bulbosum* L.), which are short-lived grass types that are similar in form and phylogenetic relatedness to cultivated barley (*Hordeum vulgare*). Theses grasses are all grouped under the subgenus *Hordeum*, while other species of the genus *Hordeum* belong to subgenus *Hordeastrum* (Blattner, 2018). Morphologically, these wild grasses exhibit variations in leaves, stems, spikes, awns, and grain size (Badr et al., 2000). Wild barley and bulbous barley are considered the closest relatives to barley and can form fertile hybrids. On the other hand, barley grass is also capable of hybridisation with barley, but the resulting offspring are infertile.

Recent studies from Turkey, Iran and Israel have emphasised the importance of these alternative hosts within the fertile crescent. Net blotch isolates with both net and spot symptoms found in barley (*H. vulgare*), wild barley (*H. vulgare* ssp. *spontaneum*) and barley grass (*H. murinum* ssp. *glaucum*) were determined to be important for the maintenance and distribution of the pathogenic population (Çelik Oğuz & Karakaya, 2021; Ronen et al., 2019; Vasighzadeh et al., 2022). Further, these studies found that *P. teres* alternativeancillary hosts harboured pathogenically relevant forms capable of producing strong virulence on different barley varieties (Ronen et al., 2019). This suggests that that cross-host

infection within the fertile crescent is possible, and phylogenetically related host species are important reservoirs to the disease.

In Australia, barley grass (*H. murinum* ssp*. leporinum*) a closely related subspecies of barley grass (*H. murinum* ssp*. glaucum*), commonly grows alongside cereal crops and acts as an alternative host for *P. teres* (Khan, 1973; McLean, Howlett, & Hollaway, 2009). However, historically barley grass isolates have shown low levels of pathogenicity on commercial barley cultivars indicating host specialisation (Khan, 1973). There have been conflicting reports on the infectivity of *P. teres* isolates collected from barley grass on commercial barley, with some studies suggesting it exhibits low virulence on barley cultivars and conversely, reports suggesting barley grass isolates were able to successfully infect domesticated barley (Brown, Steffenson, & Webster, 1993; Kenneth, 1962). More recent studies have sought to determine the impact, genetic similarity and epidemiology of barley grass *P. teres* in Australia, and determined that barley grass isolates are not likely to significantly contribute to infections on cultivated barley (Linde & Smith, 2019; Poudel et al., 2017).

In common with the *P. teres*-barley pathosystem, there have been reports of both net form and spot form physiological variants of the disease identified on barley grass (Poudel, 2018). Attempts to distinguish between the two forms and mating types with typical marker based systems designed for *Ptm*/ *Ptt* found on barley have been unsuccessful (Lu, Platz, Edwards, & Friesen, 2010; Poudel, 2018), with barley grass isolates found to exclusively amplify *Ptt* mating-type markers (Poudel, 2018). This anomaly has led to the assumption that barley grass isolates are exclusively *Ptt* (Linde & Smith, 2019; Poudel, 2018; Poudel et al., 2017).

Due to the anomalies and inconsistencies in the literature I aim to examine the genetic diversity and population structure of *P. teres* on barley grass collected from the south-Western barley growing regions of Western Australia using DArTSeq SNP markers and compare these isolates to those found in cultivated barley. The objectives of this study are 1) to determine whether *P. teres* populations from barley and barley grass share the same population pool by investigating genetic relatedness among host-associated populations, and 2) characterise *P. teres'* population genetic diversity and structure of on both barley and barley grass, in nearby, and amongst barley fields in Western Australia. To determine this, we collected symptomatically consistent net-blotch samples from both barley grass and barley within Western Australia, finding that there is likely little genetic crossover between the diseases from separate hosts, suggesting genetically isolated populations. Furthermore, we found a lack of clear population structure within hosts between sample locations, indicating a lack of clear geographical structure that may be a product of low genetic diversity or the presence of frequent outcrossing and migration between regions.

Methods:

Isolate collection

To obtain isolates for this study, fifteen locations from six localities within the Western Australian (WA) barley growing regions were sampled for disease lesions consistent with barley net blotch. Of these locations, thirteen were barley grass (*H. murinum* ssp. *leporinum*) sites and two were barley (*H. vulgare*) fields. Barley samples were collected nearby to barley grass collection sites. Diseased lesions from each host were visually classified as either spot form or net form (Figure 4—1) and samples stored in paper envelopes to dry for up to two weeks. Additionally, four barley isolates were added to the analysis; *Ptt* type isolates W1-1 and NB29, *Ptm* type isolate SG1, and barley isolate 17-058. Barley isolate 17-058, first isolated in Victoria in 2017 and recorded as causing spot form symptoms and was included this study as it had previously been shown to show strong genetic similarity to barley grass isolates collected in Western Australia in 2020 (20BG2001, 20BG2004, 20BG2005) (Hassett et al.,

2023). Despite being isolated from barley, for this study it was considered a barley grass isolate due to its phylogenetic and pathogenic similarity to other barley grass isolates (Table 4-1).

Figure 4—1. Spot form and net form symptoms on barley grass (*H. murinum* ssp. *leporinum*). Isolates displaying symptoms similar to the left-hand leaf were considered net form isolates and isolates showing symptoms consistent with the right-hand image were considered spot form isolates.

DNA extraction and Genotype-by-sequencing

Eighty-five barley grass and thirty barley mono-conidial isolates were obtained from approximately 200 symptomatic leaf samples using the method described in chapter two (Hassett et al., 2023). From each of these isolates, DNA was extracted from cultures incubated in Fries 2 liquid medium for seven days (Acharya et al., 2013; Fries, 1938). Mycelia was then recovered from the tubes and processed using Wizard Genomic DNA Purification Kit (Promega, Fitchburg, Wisconsin, USA) following the manufacturer's 'Isolating genomic DNA from plant tissue' protocol. A NanoDrop spectrophotometer (Thermo Fisher Scientific, MA, USA) was then used to adjust DNA concentrations to >100 ng/µl to suit DArTSeq requirements. Additional DNA from each isolate was used to ascertain the mating type and formae using the polymerase chain reaction (PCR) method described by Lu et al. (2010).

DArTseq genotyping was performed as described in chapter two; Hassett et al. (2023). In total 3733 DArTSeq SNP markers were produced. Single nucleotide polymorphism (SNP) markers were converted into the Genalex file format (Peakall & Smouse, 2012) and imported into RStudio 4.2.0 (RStudio, Boston, MA, USA). A >10 % missing data cut off was then applied, filtering out both markers and isolates bellow the given threshold. Phylogenetically uninformative markers were then removed using a percentage of divergent isolates as a cut off (cut off = 2/*n*) and minimum allele frequency (MAF = 0.01), the *informloci* command in the *poppr* package (Kamvar, Tabima, & Grünwald, 2014).

Identification of multi-locus genotypes

To account for potential genotyping errors associated with DArTSeq, which may result in isolates being placed in different multi-loci genotypes (MLGs), a cut-off of sample similarity was determined by first establishing the genetic distance between two barley grass replicates (20BG2001). The genetic distance between the replicates was calculated using the *provesti.dist* command, and applied to classify isolates sharing a genetic distance below a certain threshold with the *mlgfilter* command in the *poppr* v 2.8.3 (Kamvar et al., 2014) Isolates which shared lower genetic than the given value were considered the same MLG. These were classified as clones and removed from subsequent analyses using the *clonecorrect* function in *poppr* v 2.8.3 (Kamvar et al., 2014).

Genetic analysis, linkage disequilibrium, and AMOVA

Genetic diversity indices for groups of isolates (forms/ types and hosts) were generated within *poppr* v 2.8.3 (Kamvar et al., 2014) using the *poppr* function. Indices were determined by comparing the number of MLGs to the expected number of MLGs (eMLG) in the original data and calculating Simpson's corrected index ((N/ (N - 1)) λ) of MLG diversity, Nei's unbiased gene diversity index, and the Shannon–Wiener index of MLG group genotypic diversity. The extent of random mating occurring within populations was determined by calculating gametic equilibrium using the standardised index of association ($\bar{r}_d$), which is independent of sample size (Agapow & Burt, 2001).

Genetic variation both within and between isolate groups (between forms/ types and hosts), was determined using an analysis of molecular variance (AMOVA) alongside population differentiation statistics, calculated using the *poppr.amova* command in the *poppr* 2.8.3 (Kamvar et al., 2014) package. Pairwise PhiPT, to measure of population differentiation, was calculated for clone corrected groups within GenAlEx 6.5 with 999 permutations (Peakall & Smouse, 2006, 2012).

Genetic clustering and subdivision analyses

The genetic clustering of isolates MLGs was determined though the multivariate statistical approach, principal component analysis (PCA. Genetic subdivision was then inferred through the use of discriminant analysis of principle components (DAPC) (Jombart, Devillard, & Balloux, 2010). PCA was performed by applying the *glpca* function in the *adegenet* package (Jombart et al., 2010). DAPC analyses were then run using the *dapc* command, based on the cross-validation function *Xval.dapc* result to retain the correct number of principal components (Dray & Dufour, 2007).

Phylogenetic tree construction

To construct phylogenetic trees, the genetic relationship between clone corrected barley grass and barley MLGs was determined using *prevosti.dist* command in *poppr* v 2.8.3 (Kamvar et al., 2014). Branch support values were calculated with 1000 bootstrap replicates in the *poppr* v 2.8.3 using the *aboot* command. The Figure 4—4 dendrogram was linked to collection locations within southern WA via the *phylo.to.map* command in the *phytools* v 1.9.16 package in RStudio (Revell, 2012).

Spore collection and detached leaf assays (DLAs)

Six isolates, BG_NBA_15A, BG_NBA_15F, and 20BG2001 from barley grass, and SG1, W1-1, and 17-058 from barley were selected to compare symptoms on barley. Conidia were harvested collected and their concentration adjusted to 2,000 spores/ml, as described in Muria-Gonzalez et al. (2023). DLAs were then performed on cultivars Baudin, Spartacus Cl and Prior. All pathogenicity tests were performed with three replicates and evaluated at seven days post inoculation (dpi), with disease symptoms scored using a modified Tekauz scale (Tekauz, 1985) as described by Muria-Gonzalez et al. (2023).

Results:

Isolate collections

A total of 119 isolates were used in this study, of which 85 were barley grass isolates from WA, 30 were cultivated barley isolates collected alongside the barley grass isolates, two were reference genome isolates (*Ptm* isolate SG-1 and *Ptt* isolates W1-1) (Syme et al., 2018). A historical WA isolate (NB29) was also included together with isolate 17-058 as a technical replicate for DArT runs (Supplemental Table 8). Of the 86 barley grass isolates 49 displayed net form symptoms on barley grass, whereas 36 showed spot form lesions. Both symptomatic types were generally found within the same area, apart from South Boscobel, where only net form was present.

Pathogenicity tests

DLA phenotyping indicated that both barley *Ptm* type isolate SG1 and *Ptt* type isolate W1-1 were virulent to moderately virulent against Baudin, Spartacus Cl, and Prior barley varieties (Table 4-1). Isolate 17-058, which was originally collected by Mark McLean as a SFNB isolate from Birchip, NSW in 2017 from an unknown barley variety, was non-virulent, producing low disease scores on the three varieties tested. Symptomatic forms of isolates SG1 and W1-1 were clearly observable, displaying SFNB and NFNB, respectively. All the barley grass isolates did not develop high enough reaction scores to determine symptomatic form, presenting as small pinpoint lesions.

Table 4-1. Pathogenicity reaction of 6 *P. teres* isolates from barley and barley grass on three barley varieties.

Isolate	Host	Form	Barley variety		
			Baudin	Spartacus Cl	Prior
SG1	Barley	Spot form	4	7	8
W1-1	Barley	Net form	8	7	4
17-058	Barley	Spot form	2	1	2
BG_NBA_15A	Barley grass	Net form	1	1	2
BG_NBA_15B	Barley grass	Spot form	1	2	2
20BG2001	Barley grass	Spot form	1	1	1

Isolates SG-1, W1-1 and 17-058 were all isolated from barley, whereas BG_NBA_15A, BG_NBA_15F, 20BG2001 were isolated from barley grass. Symptomatic forms/ types were recorded at time of isolation. Reaction results are the average disease score of three replicates for each isolate and variety tested; scores range from 1 – 10, with higher scores indicating greater disease severity.

Mating type marker validation

PCR diagnostic markers for distinguishing SFNB and NFNB of barley were used to determine whether they were effective in distinguishing barley grass isolates displaying the corresponding symptoms. However, all barley grass isolates (n = 86, regardless of symptomatic phenotype) amplified only the *Ptt* selective markers. Of the 30 barley isolates collected for this study, PCR diagnostic markers confirmed that 14 were *Ptt* (NFNB) and 16 were *Ptm* (SFNB) in accordance with their symptomatic designation.

Genetic diversity and population structure

After data filtering and clone correction, 102 multi-locus genotypes (MLGs) were found, based on 1592 SNP markers (Table 4-2). All isolate symptomatic (net and spot forms/ types) and host (barley and barley grass) groupings showed a high level of individual variation, with a low level of clonality found in both barley grass isolate symptomatic types and the barley net form. Nei's unbiased gene diversity, which represents the probability that a pair of randomly chosen marker alleles are different, was found to be moderately low for all sample groups (>0.046), indicative of low levels of genetic diversity for most groups. A high genotype diversity was found in all populations, as given by the corrected

Simpson's index ($1 - \lambda d > 0.97$). The Nei's unbiased gene diversity being suggests that the individual genetic variation within the isolates is likely low, meaning that they are likely highly related to one another. Whereas the high genotype diversity given by Simpson's index suggests that, despite this low genetic diversity, there are many individuals within the population indicating high genotype diversity, as the low level of clonality attest. Despite the high multi-locus genotype diversity, all groups appeared to be reproducing asexually as suggested by the standardized index of association ($\bar{r}d$) values; however, barley SFNB isolates may have been sexually reproducing more frequently than the other isolate groups ($\bar{r}d = 0.001$, $P = 0.27$).

Table 4-2. Genetic diversity indices for isolates sampled from barley (spot form and net form) and barley grass (spot form and net form), produced within *poppr* (Kamvar et al. 2014).

Host	Symptom	N	MLG	eMLG	$1 - \lambda$	H_{exp}	$\bar{r}d$
Barley Grass	Net	49	43	15.2	0.99	0.02	0.005
	Spot	37	29	14.0	0.98	0.03	0.111
Barley	Net	17	14	13.3	0.97	0.03	0.054
	Spot	16	16	16.0	1.00	0.04	0.001

N: The number of individuals within a sample group; MLG: The total number of identified multi-locus genotypes (MLGs) within a symptomatic form/ type; eMLG: The expected number of MLGs; $1 - \lambda$: The Corrected Simpson's index of MLG diversity, the likelihood that a pair of randomly chosen isolates from the same dataset are different genotypes; H_{exp}: Nei's unbiased gene diversity index, the probability that a pair of randomly selected markers are different alleles; $\bar{r}d$: the standardized index of association; the associated likelihood of a population's individuals freely recombining, with a null hypothesis of zero.

Total SNP variance through AMOVA of the entire clone corrected data showed significant genetic differentiation between the two host groups which accounted for most of the genetic variation (71 %, $p = 0.01$), whereas variation between samples within host groups was lower (~24 %, $p = 0.01$), and variations within sample groups (both host and symptomatic form/ type) was lowest (~5 %, $p = 0.01$). These results indicate that host differentiation likely contributes the most to genetic differentiation rather than physiological symptoms. PhiPT was highest between barley grass net form and barley SFNB populations (0.933), followed by barley grass net form and barley NFNB populations, and lowest between the barley grass net form and barley grass spot form populations (0.029, Supplemental Table 9).

PCA was used to inform genetic groupings of individual genotypes, showing three defined isolate groupings when the first two principal components were retained (Figure 4—2). The first principal component, explaining 66.1% of the variation, separating the respective hosts, with *Ptt* and *Ptm* MLGs clustering closer together than all barley grass MLGs. The second principal component, PC2, explaining 22.7% of the variation, separated *Ptt* from *Ptm* genotypes but failed to separate the two symptomatic types, spot and net forms of barley grass isolates. Isolate 17-058 segregated from the commercial barley isolates and grouped with the barley grass isolates.

Discriminant analysis of principal components (DAPC), an unsupervised multivariate clustering method, was used to assign isolates to groups of related individuals without *a priori* assumptions of host or phenotype (in which, isolate 17-058 was considered a barley grass isolate). Results also suggested three populations ($K = 3$) when two principal components are retained, separating *Ptm* from *Ptt*, with all barley grass isolates clustering together. Further, a secondary principal components analysis was performed on the putative three populations and also showed significant differentiation between barley NFNB, SFNB and a joint cluster of barley grass net form and spot form isolates. The corrected Simpson's index also suggested high genetic diversity within each cluster ($1 - \lambda d > 0.98$).

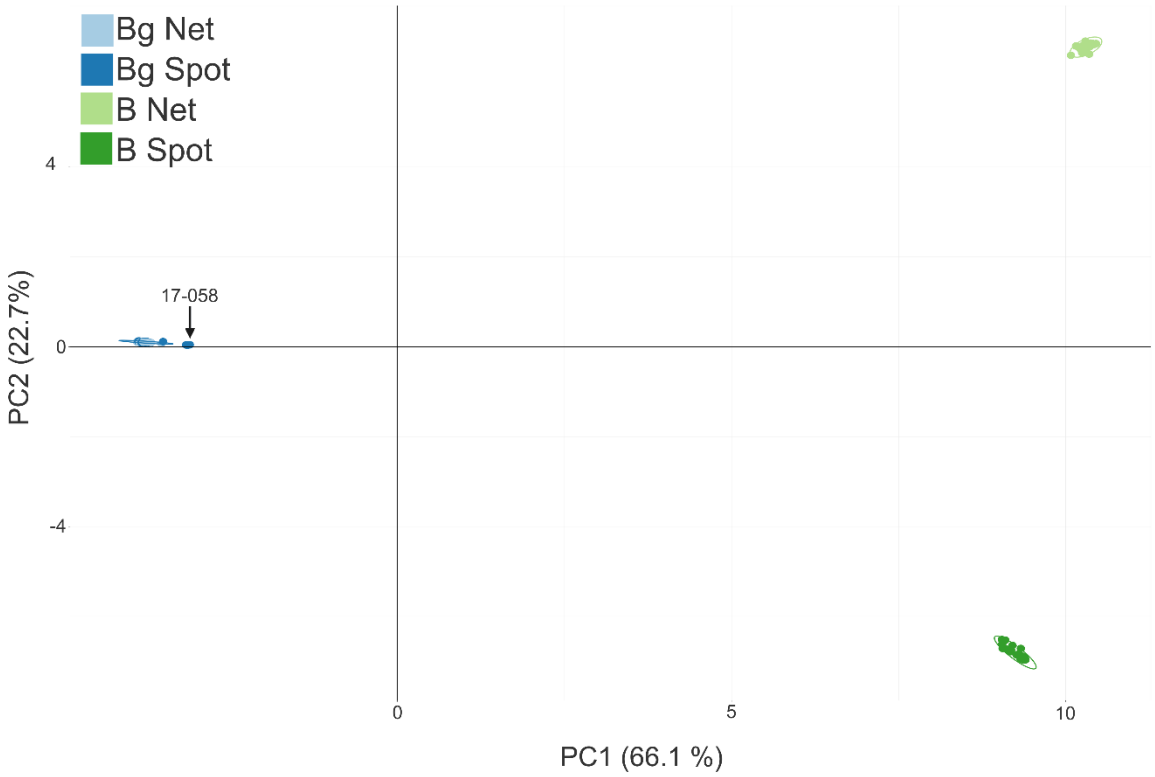

Figure 4—2. PCA of barley and barley grass multi-locus genotypes from Australia (n = 102) showing the first two principal components. Isolates were collected from spot and net form/ type symptomatic barley (B) or barley grass (Bg). Ellipses show ninety-five percent confidence intervals. Isolate 17-058 is indicated with black arrow.

To show genetic relationships between the isolate groups a phylogram was generated (Figure 4—3), which showed a lack of differentiation between barley grass MLGs regardless of their symptoms, while *Ptm* and *Ptt* isolates of barley were clearly differentiated from barley grass isolates with 100% bootstrap support, and from each other also with 100% bootstrap support. These results reinforce the inference determined through AMOVA that most of the genetic variation was determined by host rather than symptomatic form and suggest that the net and spot form isolates of barley are more closely genetically related to one another than to their respective net and spot symptomatic types in barley grass.

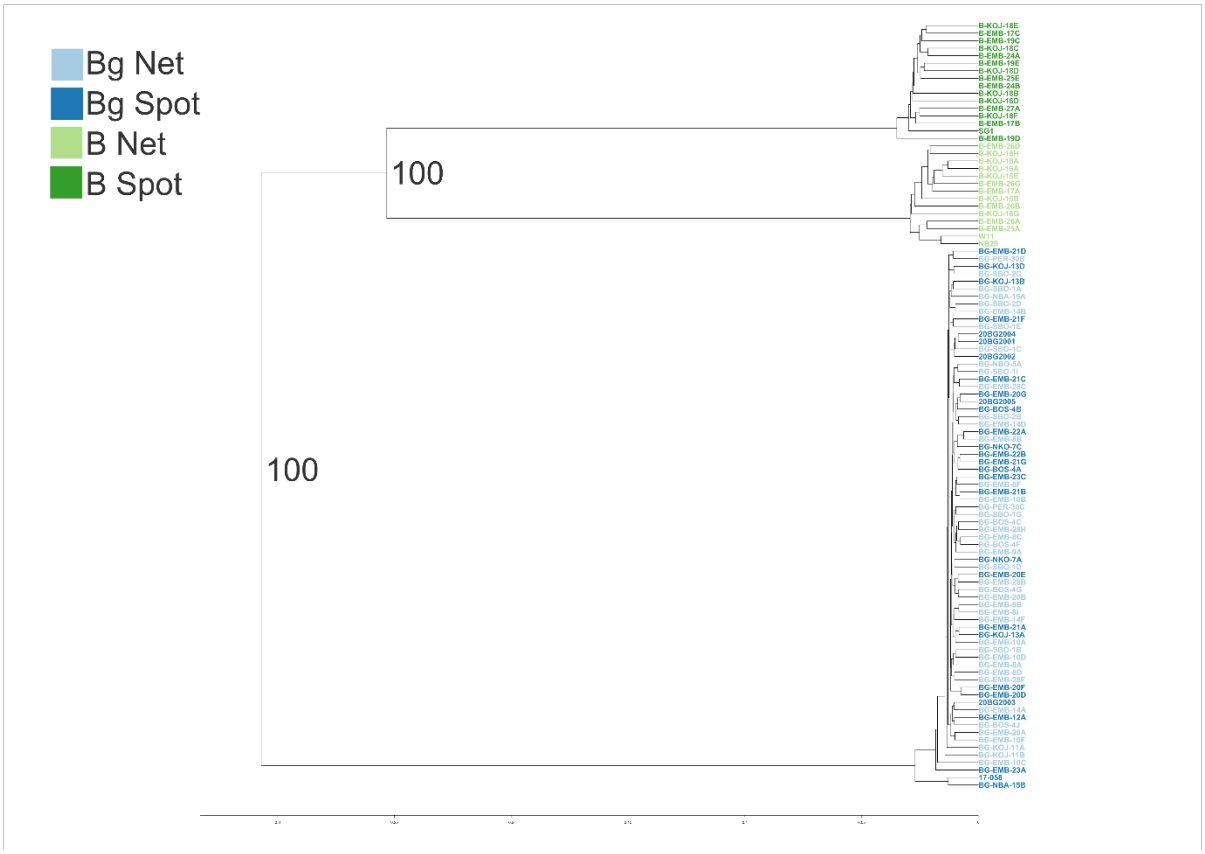

Figure 4—3. Phylogram of 72 barley grass (Bg) and 30 barley (B) *P. teres* genotypes. The dendrogram was produced using UPGMA cluster analysis, based on 1592 markers and 102 MLGs, using Prevosti's distance model and 1000 iterations (Prevosti, Ocaña, & Alonso, 1975).[#]

A further analysis into the population structure of barley grass isolates alone was undertaken to determine the genetic distribution of each symptomatic types and their respective genetic relatedness using the 71 Western Australian barley grass genotypes, removing isolate 17-058 (Figure 4—4). The lack of clear differentiation between groups, regardless of disease phenotype or locality remained consistent in the dendrogram. Further, inferences into whether symptom or location played a role in population structure determined through AMOVA suggested that most of the genetic variation was determined by individual variations within samples (~95 %, p = 0.01) with little population structure associated with location of isolates (~5 %, p = 0.01), and no population structure associated with symptomatic disease expression (>1 % p = 0.745). A lack of isolate groupings was also observed on a PCA when the first two principal components were plotted against one another (Figure 4—5). Furthermore, unsupervised clustering analysis performed without a priori knowledge of isolate host or phenotype suggested the existence of a single population (K = 1) when seventy principal components were retained.

[#] Please note a suitable outgroup species, such as *P. graminea* or *P. semeniperda*, was not available to genotype during this research.

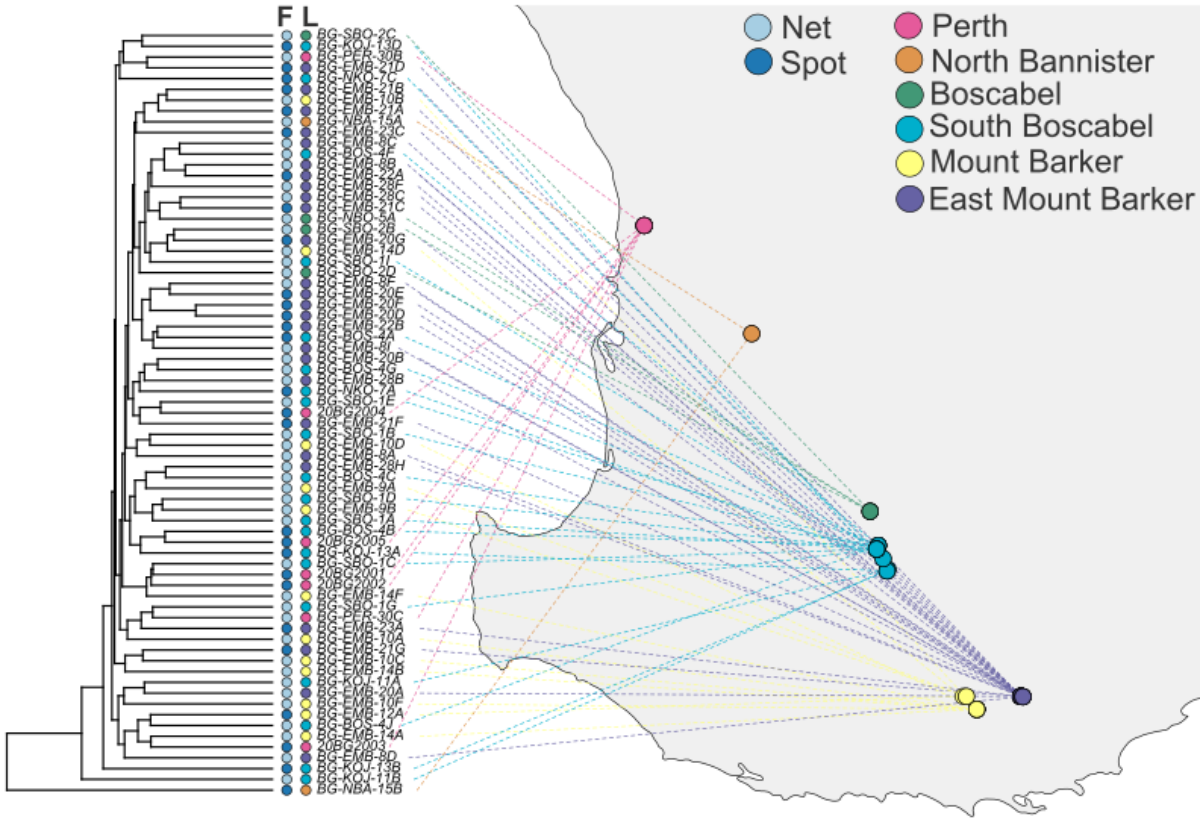

Figure 4—4. Phylogram produced using UPGMA cluster analysis based on Provosti's distance using 71 Western Australian barley grass *P. teres* genotypes. The dendrogram is based on 1592 markers and 71 WA barley grass MLGs, using Prevosti's distance model using 1000 iterations (Prevosti et al., 1975). Column F; stands for symptomatic type observed at time of collection, L; stands for locality in which the isolate was sampled.

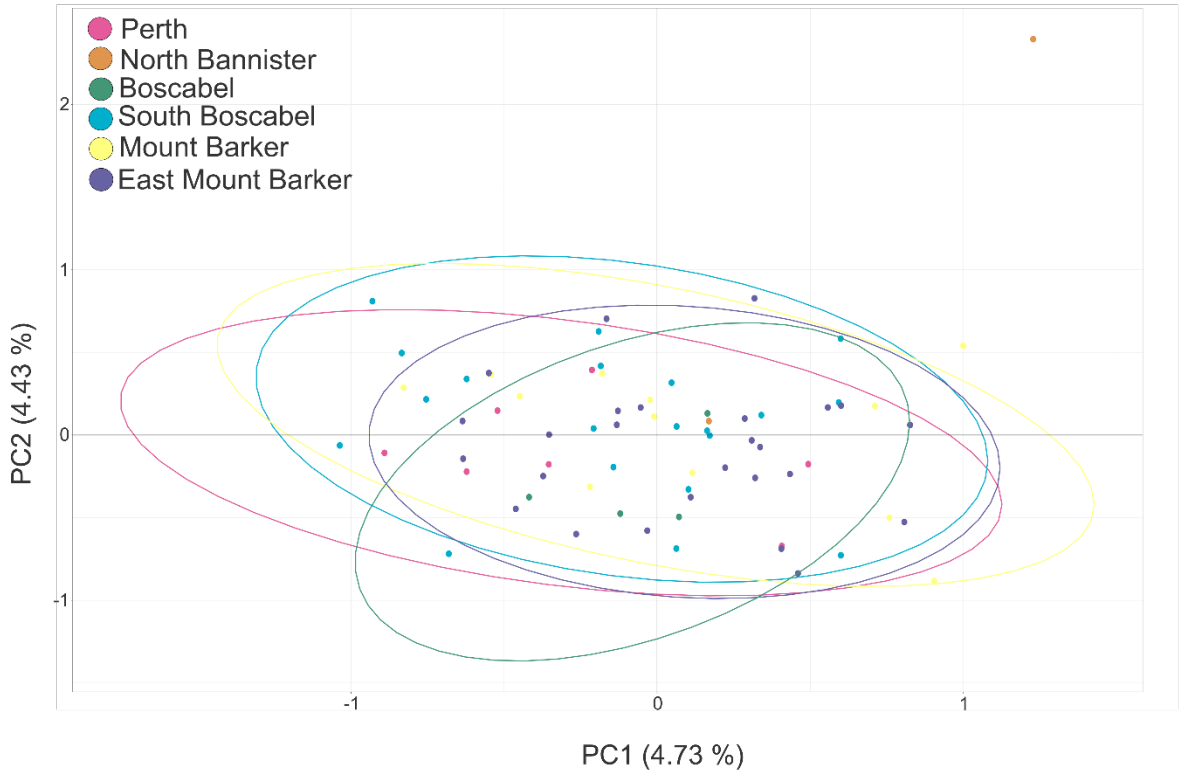

Figure 4—5. PCA of barley and barley grass genotypes from Australia (n = 71) showing the first two principal components. Isolates were collected from spot and net symptomatic types on barley grass from six general locations. Ellipses show respective ninety-five percent confidence intervals of the six localities sampled, pink: Perth; orange: North Bannister; green: Boscabel; blue: South Boscabel; yellow: Mount Barker; and purple: East Mount Barker.

Discussion:

To date, there are no published works directly investigating the genetic relationships between barley derived *Ptm* and *Ptt* isolates, and barley grass *P. teres* isolates in Western Australia. This study compares the population genetic structures, symptomatic forms/ types, pathogenicity, and diversity of isolates from Western Australia in a single study. Recent research has focused on more specific relationships such as *Ptt* and barley grass *P. teres* or lack a clear distinction of symptomatic forms/ types (Linde & Smith, 2019; Poudel, 2018). As such, this study represents the most up to date overview of the genetic relationships between barley and barley grass *P. teres* isolates in Australia, allowing for a greater understanding of their population compositions and the genetic relationships.

Host specificity and genetic relationship between *P. teres* subspecies

In this study it was found that the host is the greatest determinant of *P. teres* genetic structure in Australia, regardless of phenotype or the specific subspecies. PCA and DAPC clearly separated both *Ptm* and *Ptt* isolates of barley from *P. teres* collected from barley grass samples, along the first axis (66.1 %), then *Ptt* from *Ptm* along the PC2 axis (22.7%). This suggests that *Ptm* and *Ptt* collectively are significantly more closely related to each other than they are to *P. teres* from barley grass but are also readily distinguished as reproductively isolated symptomatic types (Yuzon et al., 2023).

Previously, primers designed for the differentiation of *Ptm* and *Ptt* (Lu et al., 2010) have been used to suggest that barley grass and barley *Ptt* isolates are likely both *Ptt* (Linde & Smith, 2019). This inference has been made due to the shared amplification of mating type primers of barley *Ptt* and barley grass isolates. Furthermore, no Australian barley grass isolates have been reported to amplify a *Ptm* mating type selective marker. Poudel (2018) suggested that lack of amplification of the *Ptm* specific mating type primers by putative spot form symptomatic barley grass isolates was a misclassification during sampling, with all isolates being net form. In contrast, these results of this study suggest that the current *Ptt/ Ptm* mating type markers are insufficient in resolving *P. teres* disease phenotypic variants from barley grass, that the amplification of *Ptt* mating type PCR products is an artifact of similar PCR primer binding-site presence and supports the concept of these variants as belonging to the same genetically autonomous group.

Lack of clear genetic differentiation of spot and net forms in barley grass.

The ongoing issues of visually differentiating spot and net forms net blotch on barley is well documented in the literature. Typically, *Ptt* develops necrotic lesions with distinct striations, developing the net-like pattern, and *Ptm* develops oval necrotic lesions with a chlorotic halo (Shipton, 1973; Smedegård-Petersen, 1971). The difficulty arises in that *Ptt* may develop pinpoint lesions on resistant host genotypes, or more spot-form-like lesions in a different host genetic backgrounds or under different to environmental factors, leading to their misidentification as SFNB (Liu, Ellwood, Oliver, & Friesen, 2010; Tekauz, 1985). Current literature has sought to rectify this issue with the use of molecular markers to differentiate both the barley *P. teres* subspecies from *Ptt*-like (mating type amplifying) barley grass isolates and their hybrids (Poudel et al., 2017), but as we have shown, the tools for this need to be developed further.

A similar field identification issue may have occurred in the case of isolate 17-058, which originally was thought to be *Ptm* as it was isolated from barley displaying SFNB symptoms. Virulence tests performed in this study suggest that, despite its origin, isolate 17-058 presented low disease reaction scores with symptoms in the form of pinpoint lesions, in common with the barley grass isolates, suggesting host resistance. Genotype data indicated that this isolate is more closely related to other barley grass isolates than it is to barley isolates. This aligns with the study performed by Linde and Smith (2019), where barley grass isolates with low disease reaction scores on barley varieties showed low genetic relatedness to barley *Ptt* and suggests that there may be occurrences, albeit infrequent, in which barley grass *P. teres* attempts to infect barley or is perhaps present as a saprophyte.

Barley grass symptomatic types do not correlate with clear genetic structure.

Despite spot-like symptoms on barley grass being described in the literature (Poudel, 2018), only the net form has been of particular focus in Australia (Linde & Smith, 2019; Poudel et al., 2017). This study sought to investigate the two symptomatic types present on barley grass by collecting isolates with both symptoms on barley grass. These isolates could not be distinguished using molecular markers, indicating that they are likely the same autonomous group. Based on this evidence it is possible that barley grass isolates in Australia represent another species of *Pyrenophora*, such as *P. graminea or P. semineniperda*. A future study using the Internal Transcribed Spacer (ITS) regions Vasighzadeh et al. (2022) and other phylogenetically informative DNA sequences of *Pyrenophora sp.* may help further clarify the relationship between the isolates of barley grass and barley.

Interestingly, barley grass isolates of the same MLG also shared the same disease phenotypes. This may indicate a genetic basis rather than an environmental factor affecting the spot and net form phenotypes; however, this is not indicative of differentiation, rather an observational correlation, as the different phenotypes may be controlled by only one or a few genes. In an international context, in California, USA, Brown et al. (1993) described infected barley grass (*H. leporinum*) as consistently exhibiting the netted lesions characteristic of infection by virulent isolates of *Ptt*, and, as with most research performed in Australia, isolates collected from barley grass exhibited reduced virulence on barley cultivars, perhaps representing pathogenic specialisation on the wild host. The issues surrounding symptomatic misidentification may be similar to that observed in the specific forms reported in barley, where immature or low-level infectivity symptoms of NFNB are often reported as SFNB, and therefore only represent a single symptomatic type being present in barley grass in Australia (Lu et al., 2010).

Lack of population structure and low genotype diversity found in barley grass isolates.

P. teres on barley grass is highly prevalent in Western Australia, being found almost as often as its prolific host. The findings of this study support the work conducted exclusively on isolates described as '*Ptt* from barley grass' paper by Linde and Smith (2019) on an Australia-wide collection, isolates used in this chapter did not cluster based on sample location, or whether they were located alongside barley fields or not. This reinforces the notion that isolates found on barley grass are genetically isolated from their *Ptm* and *Ptt* relatives on barley, and proximity to commercial crops does not alter the genetic and pathogenic autonomy.

Both alternative hosts and hybridisation between different *P. teres* forms are potential sources of new virulence gene combinations. Further, recent studies have shown that there is global movement of both *Ptt* and *Ptm* of barley, hybridising upon migration with local genotypes, bringing new advantageous mutations to local populations (Dahanayaka et al., 2021; Hassett et al., 2024). Grasses *H. murinum* ssp. *leporinum* and *H. glaucum*, found in Australia as an introduced species, are collectively known as barley grass, and are native to the Mediterranean region. Interestingly, *H. glaucum* has been implicated in harbouring genetically similar and pathogenically relevant genotypes to those found on commercially grown barley varieties in Israel (Ronen et al., 2019), which, along its surrounding countries, are at the centre of barley origin and have been described as the epi-centre of pathogen diversity (Kenneth, 1962). The *H. murinum* subspecies complex has also been described as having a high propensity for outcrossing (Booth & Richards, 2008). If breeders use exotic germplasm (land races and wild varieties) to improve commercial lines, given the genetic richness of such gene-pool, it is not unreasonable to suggest that inter-species crossing may introduce undesirable traits by accident, such as the ability for *Pyrenophora* from barley grass to infect barley. Other important relevant barley diseases, such as scald (*Rhynchosporium commune*) utilise barley grass as an alternative host of barley in Australia (Linde, Smith, & Peakall, 2016). Scald isolates found on weeds

were found to harbour virulent isolates capable of infecting cultivated barley. This suggests that the genetic divide between barley grass and commercial barley may be breached by fungal pathogens.

Recent studies have shown that that *P. teres* found on barley are capable of hybridisation (Poudel et al., 2017). Natural hybridisation between *Ptm* and *Ptt* are thought to be rare, with only a few confirmed natural hybrids being identified, but successful laboratory hybrids have been produced (Poudel et al., 2017). The pathogenicity profiles of laboratory produced hybrids by Jalli (2011) was found to be different to that of either of the parent isolates, with some cases of hybrid progeny being capable of infecting barley varieties to which both parent isolates were avirulent. No barley grass and barley *P. teres* isolates have been proven to successfully hybridise, however Poudel et al. (2017) identified a single isolate with genetic similarity to both barley and barley grass *P. teres* isolates, although hybridisation was not confirmed. This represents the possibility of hybridisation events may occur naturally, even if very rarely.

Conclusions

This study clarified that barley grass and barley *P. teres* populations are genetically isolated, and that proximity between barley grass and cultivated barley does not affect their genetic separation. The biggest factor distinguishing the populations was the host, with *Ptm* and *Ptt* of barley more genetically related to each other than isolates from barley grass. Despite both spot and net phenotypic types of *P. teres* being present on barley grass, genetic markers used in this study could not distinguish the symptomatic types as they do in *P. teres* from cultivated barley. The high degree of genetic separation between barley grass and barley isolates suggest they should be categorised as separate species.

Additional chapters notes

Given the resemblance of symptoms and fungal morphology, a high degree of relatedness was initially inferred. The species identification of the isolates were not made as most other studies have also suggested barley grass isolates are also closely related to *Ptt* and *Ptm* and within the *P. teres* clade (Linde & Smith, 2019). As mentioned in the discussion future studies may wish to define the species more clearly with the use of direct sequencing of single copy genes.

Data accessibility

This chapter was created with the intention of submitting the research and its findings as a manuscript to a peer reviewed journal at a later date. External tables found in this chapter, too large to appropriately fit in this thesis, including DArTSeq sequencing data generated from this study are available from https://doi.org/10.25917/kxg8-ba15.

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General Discussion, future recommendations and Conclusion

General discussion and future recommendations:

In chapter 2 it was observed that the genome wide coverage afforded by DArT technologies produced a diverse set of DArTSeq SNP and SillicoDArT markers, allowing for the identification of two genotypic groups in Western Australia. Genetic markers highly associated with the distinction between the two genotypic groupings implicated genomic regions which have been identified to be involved in fungicide resistance. This suggested that genetic selection of the pathogen in Australia may currently be more associated with fungicide resistance than to the variables initially tested in this study being isolate geographic location, agroecological zone or sampled field. Despite this segregation into two genetic groupings, an important finding is that, in Australia, *Ptm* shows a high degree of reproductive continuity between fields, agroecological zones, and states, at least among the two most popular susceptible varieties at the time, Spartacus Cl and RGT Planet. This indicates there is little geographical isolation nationally or cultivar-related directional selection, such as host resistances, within Spartacus Cl and RGT Planet in Western Australia, and that the pathogen is highly mobile across the country.

In the field of plant pathology and crop improvement, differential sets play a critical role in understanding host-pathogen interactions. A host differential set refers to a collection of host genotypes chosen to represent a spectrum of resistance genes or alleles. Barley differential sets are essential for identifying variability and virulence in *Pyrenophora teres* (Afanasenko, Jalli, Pinnschmidt, Filatova, & Platz, 2009). Using these sets can identify specific interactions between different barley genotypes and pathogen strains, crucial for developing and deploying host resistance genes in breeding programs aimed at creating resistant barley varieties. This approach helps in understanding the pathogen's diversity and adapting barley breeding strategies to combat evolving strains effectively.

Understanding the genetic diversity and population structure of plant pathogens like *Ptm* are a necessity in the development of targeted and effective disease management strategies. By identifying hotspots of genetic diversity or regions with high pathogen dispersal, farmers and agronomists can implement more tailored approaches for disease management and control, such as deploying resistant cultivars or adjusting fungicide application practices. This study revealed that *Ptm* is a genotypically diverse highly mobile pathogen within Australia, underlying the need for nationally coordinated disease management effort and there is a need for resistance breeding strategies for effective future disease management. The challenge to breeders in developing new varieties will be in extensive testing against the range of known pathotypes and in deploying resistance genes combinations that reduce the likelihood of their breakdown.

Moving to a higher scale in chapter 3, the evaluation of the population structure and genetic diversity of *Ptm* at an intercontinental level using DArTSeq markers, revealed the complex interrelationships the genotypic groups possess. Genetic diversity and population structure were found to be significantly different to that of *Ptt* (Dahanayaka et al., 2021); population structure was found to be more regionally linked for *Ptm* than that of *Ptt*.

Overall, the results uncovered population subdivision related to country of origin and, except for Turkey, similar amounts of diversity within countries as among countries. Regional selection of *Ptm* was also evident, particularly in the case of fungicide resistant isolates. Interestingly, isolates from Hungary, the RSA and Australia appeared to be the most inter-regionally genetically related. A set of Western Australian isolates, which in chapter 2 was shown to be genetically separated from the bulk

of Australian isolates, showed a high degree of relation to isolates from the RSA. This suggested that there may have been a recent migration of RSA-like isolates into Western Australia. Evidence from the analysis performed in chapter 2, whereby some of the most significant markers associated with differentiating genetic groupings were found in the *CYP51A* gene, encoding the target of DMI-fungicides, the genetic variability and fungicide resistance profiles of both, the Australian and RSA isolates, was investigated to better understand the potential origin of fungicide resistance in *Ptm* in Australia. It was found that Australian isolates with a high degree of whole genome similarity to RSA isolates also had very similar resistance profiles against the DMI-fungicide, tebuconazole, and shared genetic characteristics around *CYP51A*. This suggests that the high-level DMI-fungicide resistance of some Australian *Ptm* isolates may have been acquired from an international origin, rather than an isolated independent evolution. The rapid integration of high-level DMI-fungicide resistance into Australian genotypes represents the robust adaptability of *Ptm*. Further, the lack of population disease structure in Australia suggests that foreign-acquired or locally developed advantageous traits are likely to spread to all growing regions within Australia unimpeded (Hassett et al., 2023).

Human trading and cultivation activities have profoundly impacted the distribution and dispersal of plant pathogens, a process that has accelerated in the current era of globalised transport (Bebber, Holmes, & Gurr, 2014; Sotiropoulos et al., 2022). Further, the identification of intercontinental migration occurring in this study suggests that the quarantine measures used in Australia may be insufficient in controlling the integration and spread of alien disease variants, hence, regular monitoring and genetic characterisation of *Ptm* populations using an appropriate marker system, such as DArTSeq, should be undertaken. Periodical monitoring and genetic characterisation along with the phenotypic assessment, namely virulence and fungicide resistance, of disease populations in Australia should be conducted to reveal potential temporal changes of the population at a genetic and phenotypic level.

Finally, in the last part of the project (chapter 4), the genetic relationship between *P. teres* found on barley grass and that found on cultivated barley in Western Australia was analysed using DArTSeq technology. It was found that, despite barley grass isolates displaying both forms of barley net blotch symptoms, the two forms of barley grass isolates were more genetically similar to each other than to their corresponding forms isolated from cultivated barley, *Ptt* and *Ptm*, suggesting a strong host selection bias. Further, net and spot symptoms of *P. teres* on barley grass did not appear to be strong determinants of population genetic structure. This highlights the need of complementing morphological observations to identify *Pyrenophora* species with appropriate molecular markers to characterise fungal cultures. Future studies should examine further the relationship between *Pyrenophora teres* of barley and *Pyrenophora* of barley grass to gain a greater understanding of their currently shared species classification.

Although the genetic separation of *P. teres* from barley and barley grass was clear in this study, there are reasons to monitor whether this host barrier remain consistent in the future. The competitive advantage afforded by mostly unmonitored hosts such as barley grass, close by to commercially grown barley varieties could give diseases such as SFNB and NFNB a ‘green bridge’ to provide primary inoculum to seedlings and a sanctuary from control measures such as fungicide applications. Concerns can be raised due to evidence of highly related hosts in the fertile crescent hosting barley-virulent varieties of *Ptm* as well as hybridisation within the *P. teres* (Çelik Oğuz & Karakaya, 2021). This may mean that, given the right circumstances, genotypes of *P. teres* may hybridise, providing genetic mechanisms to infect both barley and barley grass or the inadvertent integration of novel virulence genes into barley infecting isolates.

Therefore, periodical monitoring and genetic characterisation along with phenotypic assessment of *Ptm* populations in Australia should be conducted to reveal potential changes of the population. This

would inform the effective deployment of strategies to counteract the changing population prior to it becoming an uncontrolled issue. Due to the migration capacity and genetic variability of the disease internationally, it is likely that significant disease pressures developed using plant resistance integration or fungicide susceptibility in the future should be challenged on a diverse panel of national and international isolates to determine subsequent efficacy of the measure. This would give an indication as to whether the disease can overcome current control measures, or potentially serve as an early warning if international isolates are found to provide an unfavourable outcome.

Conclusions

This study provides novel and highly valuable knowledge for understanding the complex *P. teres*-barley pathosystem. Further, the results presented in this thesis have proven the effectiveness of deploying a genome-wide marker system to understand the population structure and genetic diversity of *Pyrenophora teres* f. *maculata*. DArTseq™ markers allowed the identification of subpopulations in Western Australia based in genomic differences associated with fungicide resistance. Our results also reported the existence of population substructure occurring within Turkey and a high genetic relatedness between the sub-structured Western Australian and South African populations. A closer analysis into the South African and Western Australian subpopulations highlighted the potential origin of the change in DMI-fungicide profiles of *Ptm* in Australia. Further, results suggest the potential for rapid evolution and global migration of the pathogen in response to changes in environmental conditions and emphasises the to develop resistant barley varieties in order to withstand future outbreaks rather than the current reliance on chemical control measures which are rapidly broken down as a result of rapid pathogen adaptation.

References.

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Bebber, D. P., Holmes, T., & Gurr, S. J. (2014). The global spread of crop pests and pathogens. *Global Ecology and Biogeography, 23*(12), 1398-1407. doi:https://doi.org/10.1111/geb.12214

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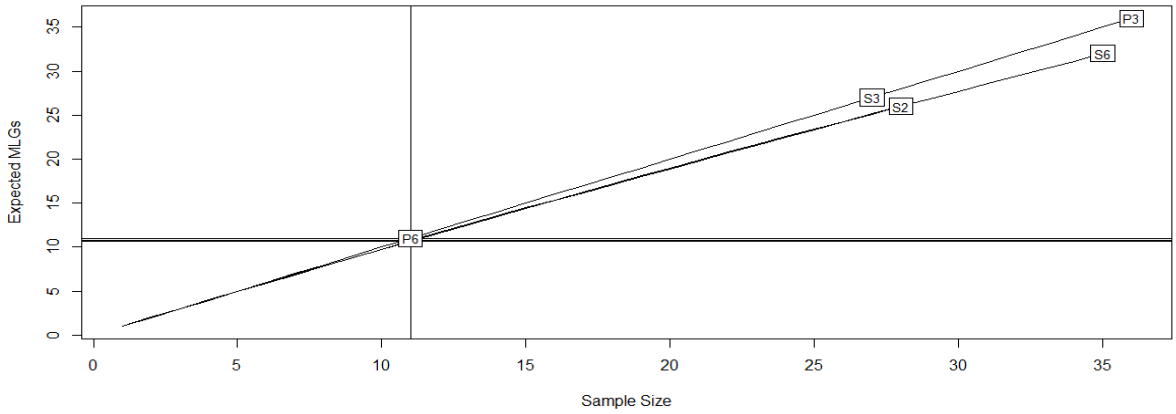
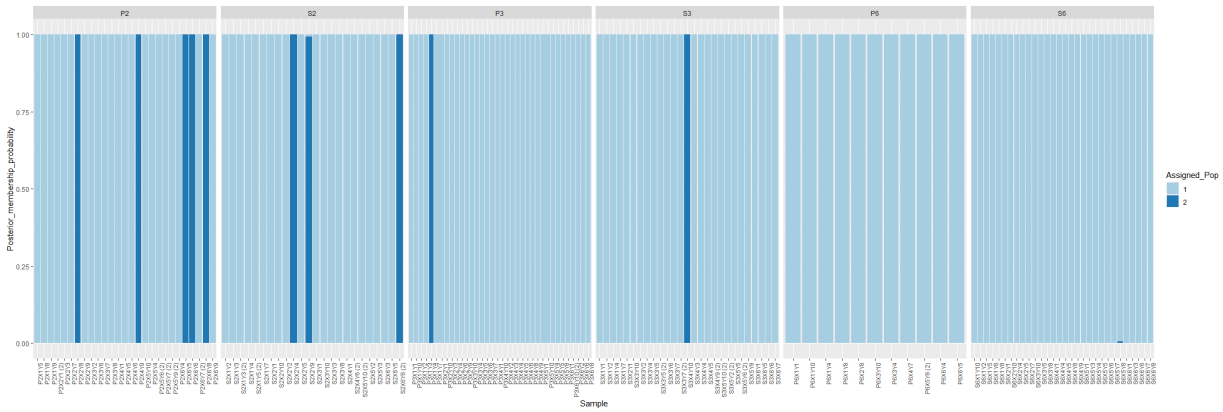
Appendix

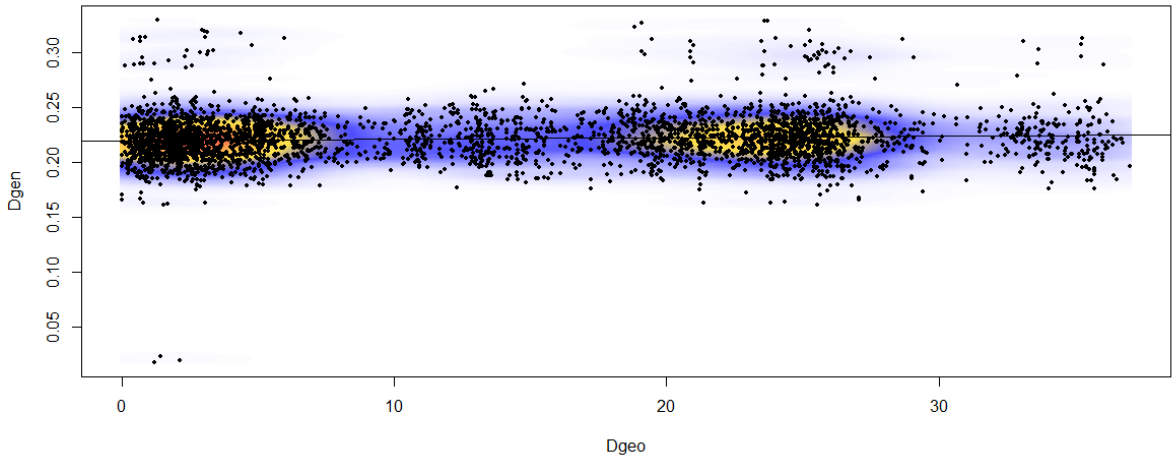
Chapter 2 supplementary files:



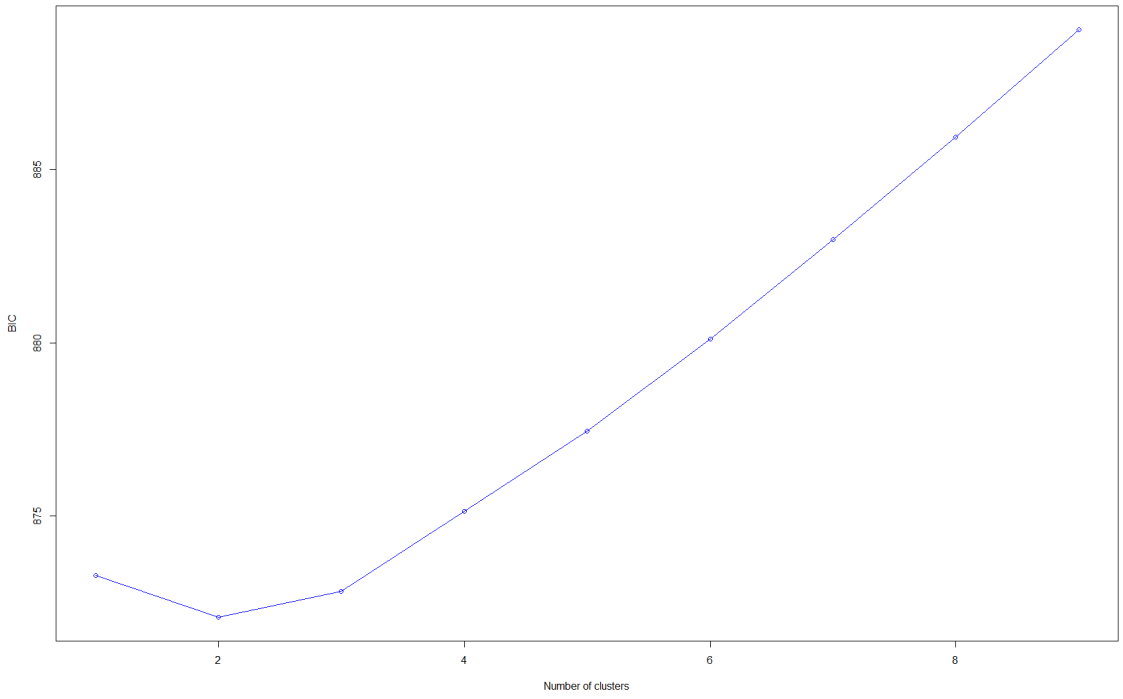
Supplemental Figure 1. Rarefaction curve analysis describing observed *Ptm* MLG richness. The plot shows the number of observed multi-locus genotypes from the six WA *Ptm* field collections compared to the expected number. Sites P2 and S3 have an identical number of MLGs and samples (Table 2-1) and are labelled at the same position. The straight lines indicate that sampling did not achieve saturation of the potential number of MLGs. Plot rendered in RStudio (RStudio, Boston, Massachusetts, USA) using the '*rarecurve*' command in R package '*vegan*' package.



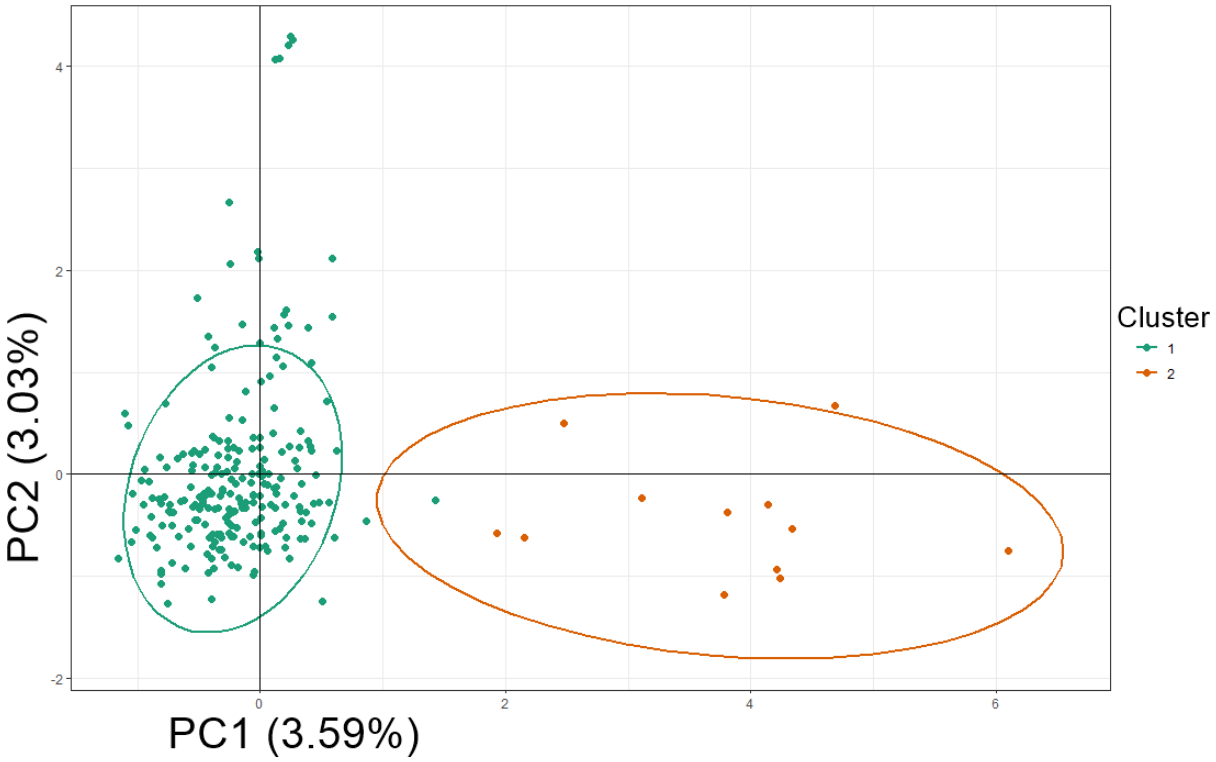
Supplemental Figure 2. Membership probability plot showing the population assignment for each of the 155 field isolate MLGs and their respective collection sites. Each genotype is represented by a single vertical bar, typically broken into coloured segments, with lengths proportional to membership of K inferred genetic clusters based on DAPC (although the samples above are predominantly assigned to a single cluster). The plot was produced in RStudio (RStudio, Boston, Massachusetts, USA) using DAPC population assignment data and the '*compoplot*' command as part of the '*adegenet'* package.



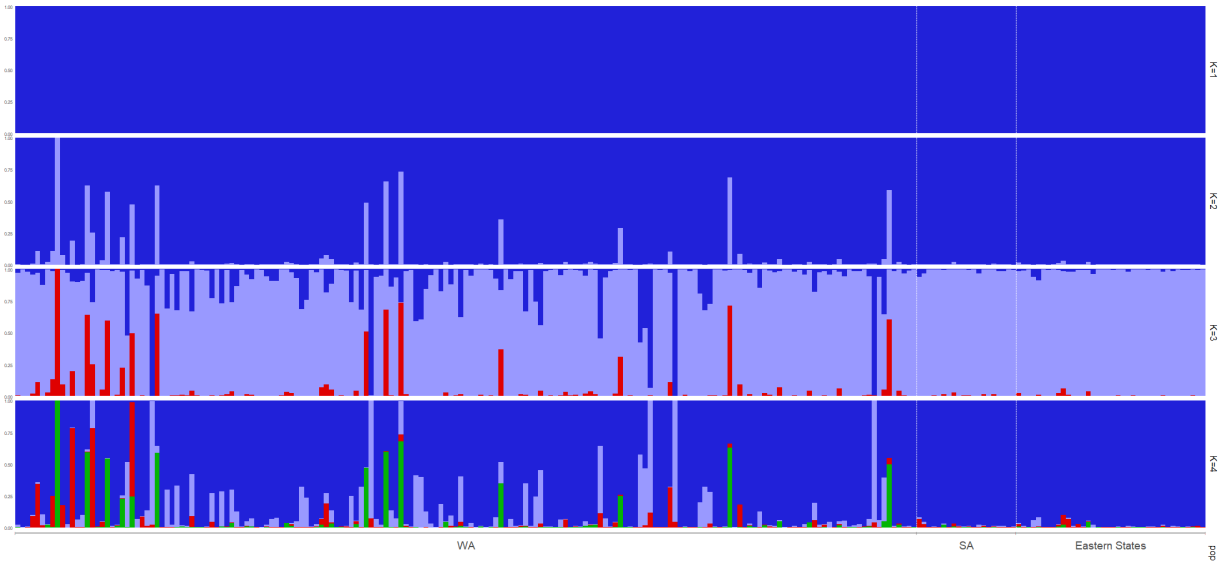
Supplemental Figure 3. Isolation by distance plot illustrating the pattern of genetic differentiation among *Ptm* genotypes collected from four Australian regions. Geographic distance is plotted on the x-axis and genetic distance is plotted on the y-axis. A low but significant amount of correlation of genetic distance with geographic distance is described by the black line (p = 0.024 and r = 0.073). The plot was produced in RStudio (RStudio, Boston, Massachusetts, USA) using the '*mantel.rtest*' command in the '*ade4'* package.



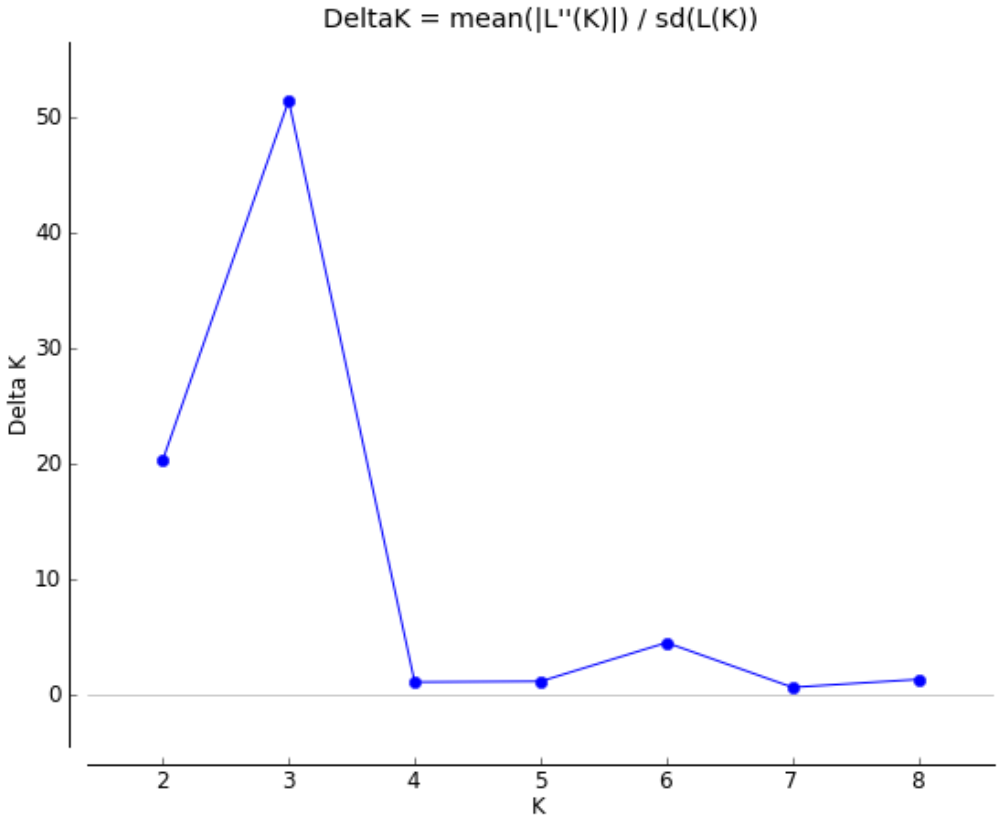
Supplemental Figure 4. Discriminant analysis of principal components (DAPC) cluster analysis of Australian *Ptm* isolates. DAPC (Jombart *et al*. 2010) was performed using 239 *Ptm* genotypes and 1271 SNP markers from both the field and regional collections. In order to produce the line plot, successive runs of K-means are recorded with an increasing number of clusters (K), after transforming the isolate SNP data using a principal component analysis (PCA). For each cluster, a BIC (Bayesian information criterion) value is computed and the optimal K value inferred from the 'elbow' inflection, here depicted as two. The procedure was performed in RStudio (RStudio, Boston, Massachusetts, USA) using the 'kmean' function as part of the '*stats*' package.



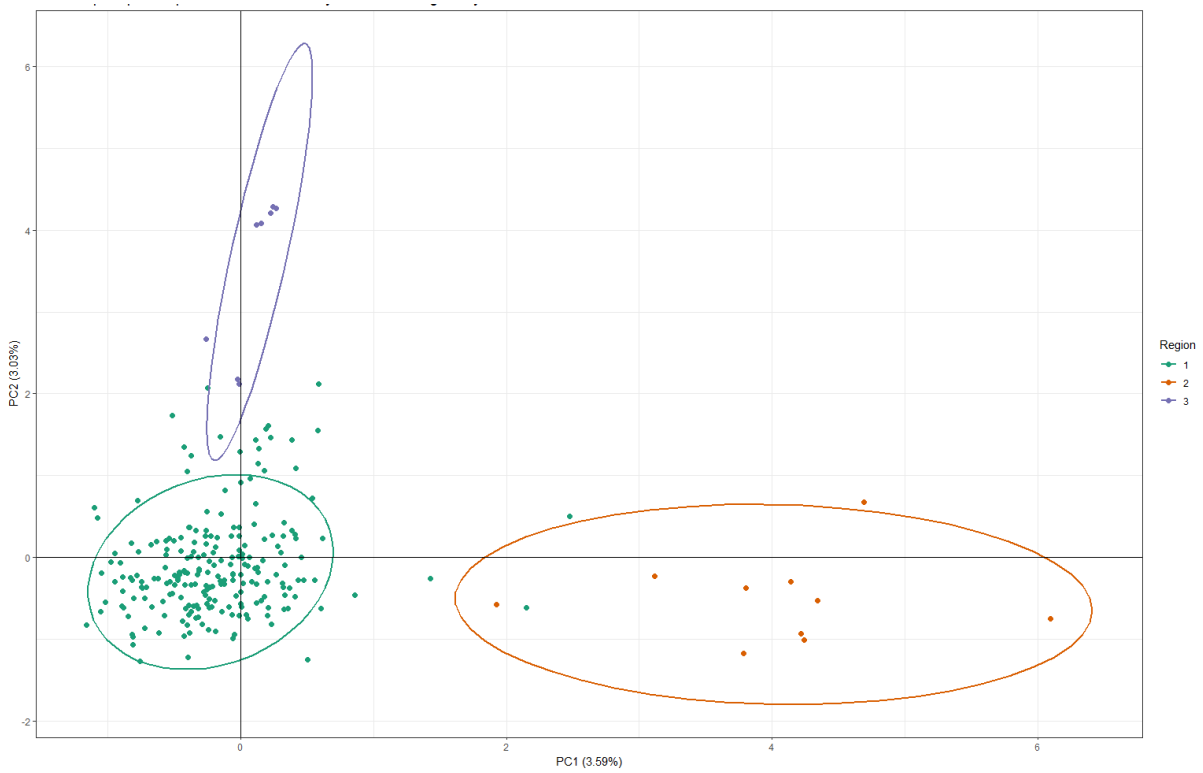
Supplemental Figure 5. Scatter plot of the first two PCA principle components resolving Australian *Ptm* isolates. The plot shows the two genotypic clusters identified by DAPC in Supplemental Figure 4. The clusters are represented by green and orange dots with 95% confidence intervals indicated by respective coloured ellipses. Figure produced in RStudio (RStudio, Boston, Massachusetts, USA) with PCA scores generated using the '*glPCA*' command in the adegenet package.



Supplemental Figure 6. Bayesian cluster analysis of Australian *Ptm* genotypes. STRUCTURE 2.3.4 (Pritchard *et al*. 2000) output depicting genotypic grouping inference based on 239 Ptm genotypes and 1271 SNP markers from both the field and regional collections. K = 1-4 is depicted, with each genotype represented by a single vertical bar broken into coloured segments, with lengths proportional to membership of K inferred genetic clusters. K = 2 was supported by DAPC in Supplemental Figure 4. K = 3 was supported by the Evanno Δk method in Supplemental Figure 7. Isolates are grouped by geographic region on the x-axis and 'Eastern States' isolates refers to isolates from Victoria, Queensland and NSW.



Supplemental Figure 7. Structure Harvester cluster analysis of Australian *Ptm* isolates. The online software Structure Harvester (Earl and von Holdt 2012) was used to determine the most likely number of subpopulations based on Q-value outputs from STRUCTURE v 2.3.4 using the Evanno Δk method (Evanno, *et al*., 2005). The most optimal number of clusters inferred in this model is three and is based on 239 Ptm genotypes and 1271 SNP markers from both the field and regional collections. The Evanno Δk method uses the greatest change in magnitude of the second-order rate of change in ln Pr(X|K) against successive K values to indicate the most optimal K value (Gilbert, 2016).



Supplemental Figure 8. Scatter plot of the first two principle components based on data from STRUCTURE 2.3.4. Structure Harvester (Earl and von Holdt 2012) indicated K = 3 as the most likely number of genetic clusters (Supplemental Figure 7). The clusters are represented by green, orange and purple dots, with 95% confidence intervals indicated by respective coloured ellipses. Image rendered in RStudio (RStudio, Boston, Massachusetts, USA) with PCA scores produced using the '*glPCA*' command in the adegenet package.

Supplemental analysis

Data analysis and quality control method was similar to that performed with the DArTSeq SNP data, with markers retained with a minimum reproducibility score of one and removing markers with >5% missing data. Following filtering, 1097 markers remained from 6321.

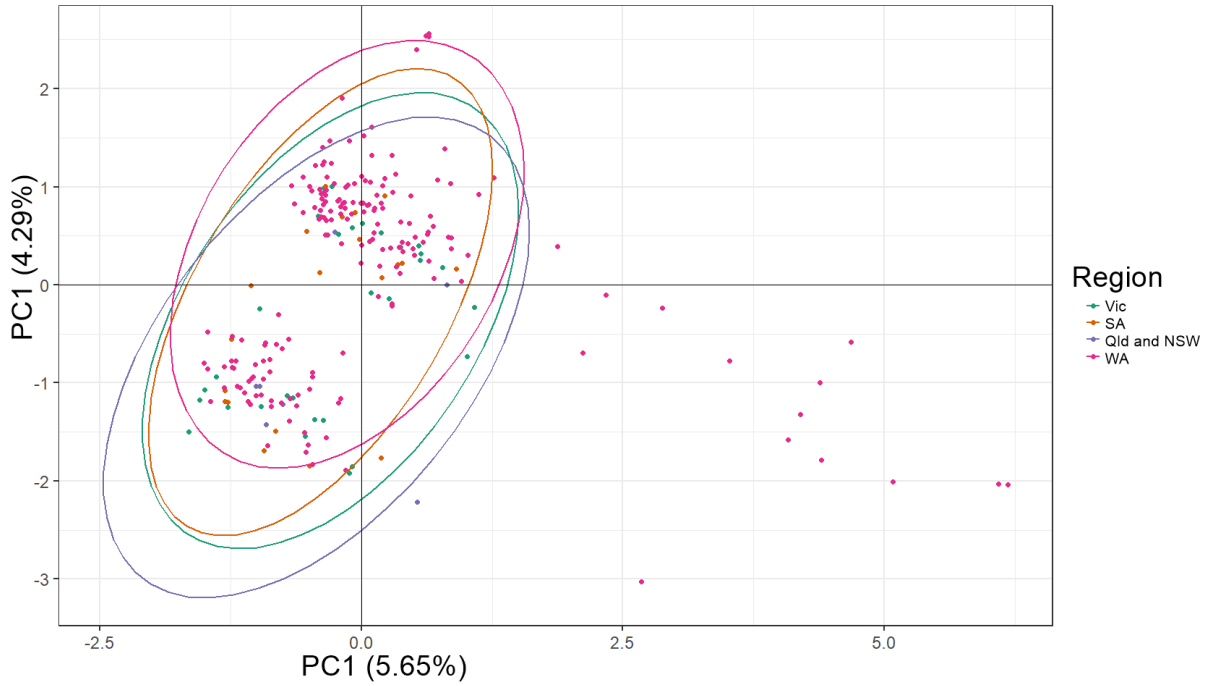
The same isolates used in the DArTseq SNP analysis were used in the sillicoDArT study. Two hundred and fifty-one isolates were classified into 239 MLGs as with the DArTseq SNP markers. All MLGs were found unique to a single state and only WA was found to consist of isolates sharing an MLG (Supplemental analysis table 1). The sillicoDArT analysis also supported the DArTseq SNP genetic diversity tests and revealed most of the genetic variation (~98%) occurred within regions whereas variation between regions was low (~1.4%), with variations between fields contributing least to genetic variation (~0.6%). The silicoDArT markers also suggested high genotypic diversity but overall isolates were closely related, supporting the DArTSeq SNP marker data.

Supplemental analysis Table 1. Genetic diversity of 251 *Ptm* isolates collected from across Australia based on sillicoDArT markers. Summary statistics were produced with the poppr command in poppr package in RStudio (RStudio, Boston, Massachusetts, USA).

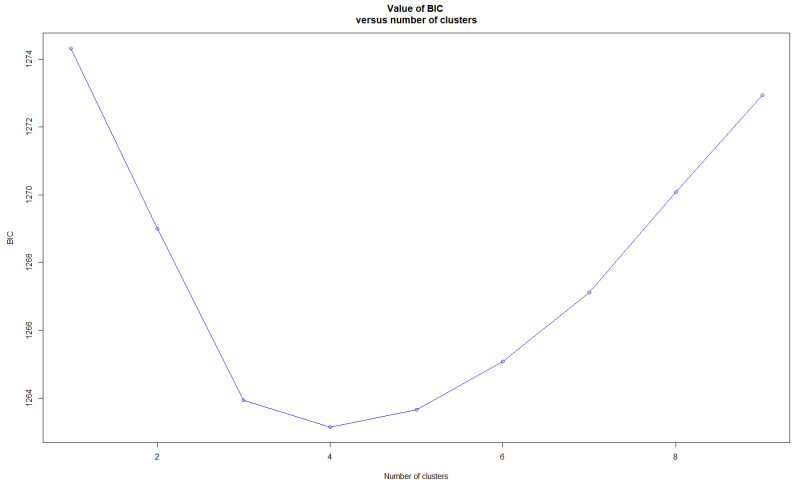
Region	***n***	**MLG**	***H***	**1-λ**	H_{exp}	$\bar{r}_d$
Vic	30	30	3.4	1	0.188	0.012
SA	20	20	3	1	0.175	0.013
Qld and NSW	8	8	2.08	1	0.193	0.005
WA	193	181	5.15	0.993	0.189	0.015
Total	251	239	5.44	1	0.190	0.013

n: Number of isolates in a sample group after data quality filtering.
MLG: The total number of unique multi-locus genotypes (MLGs) identified per region. *Indicates the cumulative number of MLGs irrespective of region.
H: Shannon-Wiener index of MLG group genotypic diversity, a measure of the number of unique genotypes and their homogeneity.
1- λ: Corrected Simpson's index of MLG diversity, the probability two isolates from the same dataset are different multi-locus genotype.
H_{exp}: Nei's unbiased gene diversity index, the probability that two randomly selected alleles are different.
$\bar{r}_d$: The standardised index of association, with a value of zero for a null hypothesis a population is freely recombining.

Principal components analysis showed no observable population structure related to collection regions when the first two principal components were plotted against one another (Supplemental analysis Figure 1). However, unsupervised clustering analysis performed without *a priori* knowledge of sample location suggested four potential populations (K = 4, Supplemental analysis Figure 2). DAPC was then used to study the four putative populations with 40 PCA eigenvalues and two discriminant analysis (DA) eigenvalues being retained. Notably, three of the populations (pop1, pop3 and pop4) were present in all four regions; however, Western Australia was the only region to contain the second population (pop2). Only one Western Australian isolate from the original regional analysis (19PTX147) contributed to the pop2, the other ten were isolates from the field level analyses. Isolates in pop2 were also found in pop2 two in the field and regional combined analysis with the SNP markers.



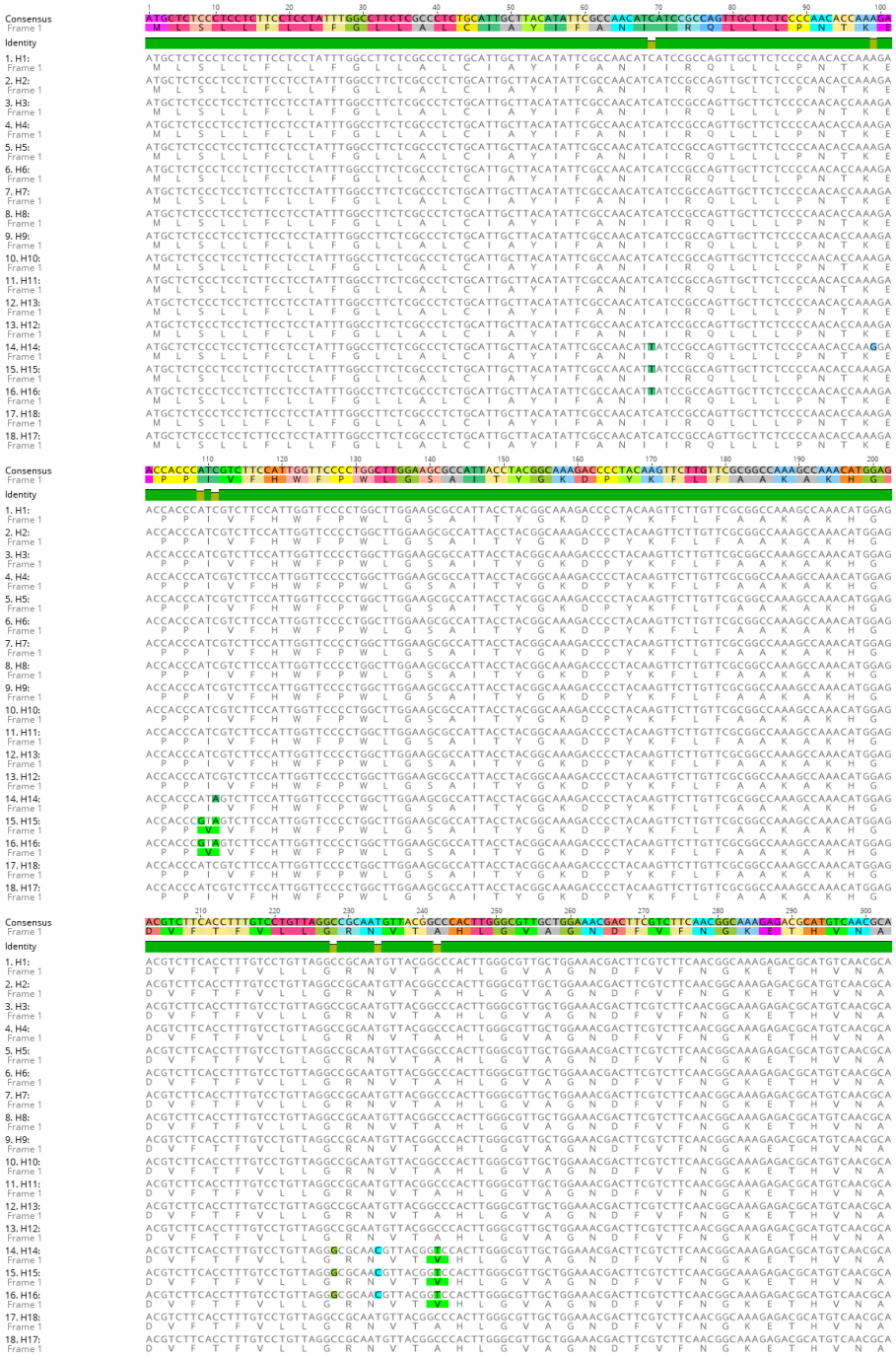
Supplemental analysis Figure 1. Principal component analysis of all *Ptm* samples from four Australian regions. The national groups are represented by purple, orange, green and pink dots, Respective coloured eclipses indicate 95% confidence intervals.

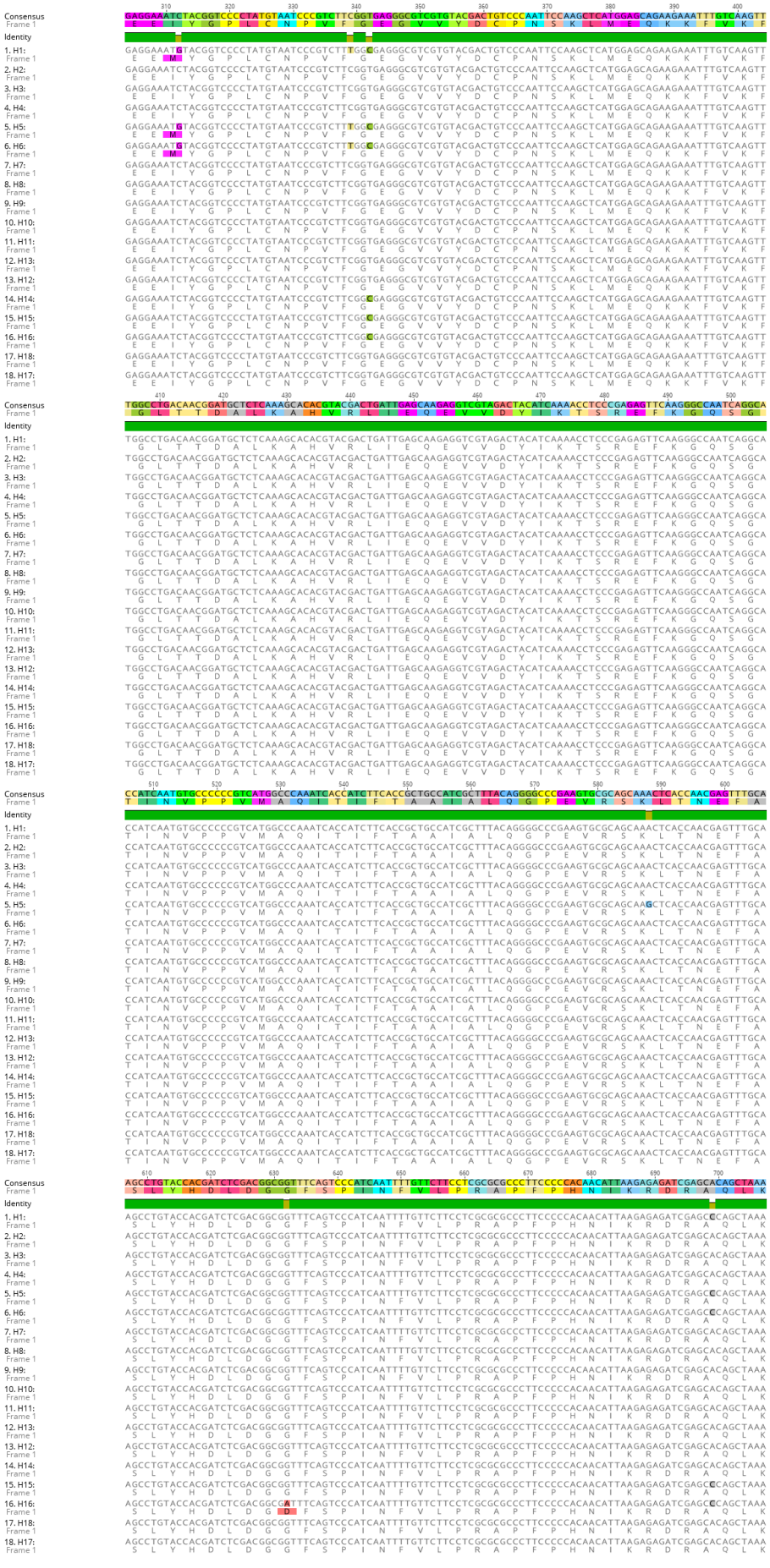


Supplemental analysis Figure 2. Cluster analysis using sillicoDArT markers based on DPAC (DAPC, Jombart et al. 2010) with 251 Australian *Ptm* isolates collected. In order to produce the line plot, successive runs of K-means were recorded with an increasing number of clusters (K), after transforming the isolate sillicoDArT data using a principal component analysis (PCA). For each cluster, a BIC (Bayesian information criterion) value is computed. Using the 'elbow method' the optimal K value was inferred as four. The procedure was performed in RStudio (RStudio, Boston, Massachusetts, USA) using the 'kmean' function as part of the '*stats*' package.

Chapter 3 Supplementary files:

Supplemental File 1. Sequence alignments of eighteen haplotypes of the *Ptm Cyp51A* gene. The alignment includes 12 *Cyp51A* alleles detected in this study (H6 – H18) and five alleles (H1 – H5) reported by Mair et al. (2020). The alignment was created using ClustalW in Geneious v R9 (https://www.geneious.com).




Consensus
Frame 1
Identity
1. H1:
2. H2:
3. H3:
4. H4:
5. H5:
6. H6:
7. H7:
8. H8:
9. H9:
10. H10:
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15. H15:
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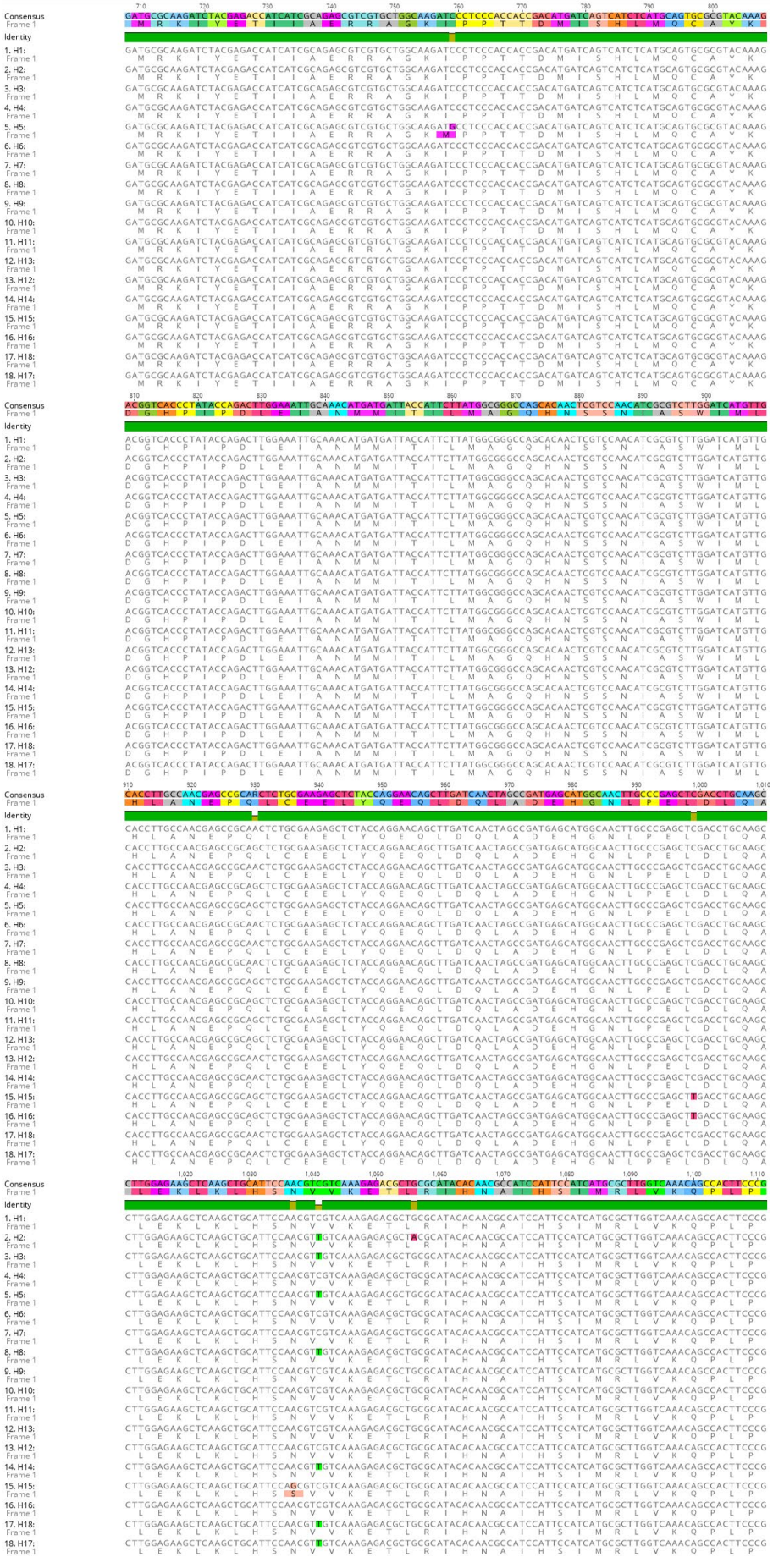
17. H18:
18. H17:


Consensus
Frame 1
Identity
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9. H9:
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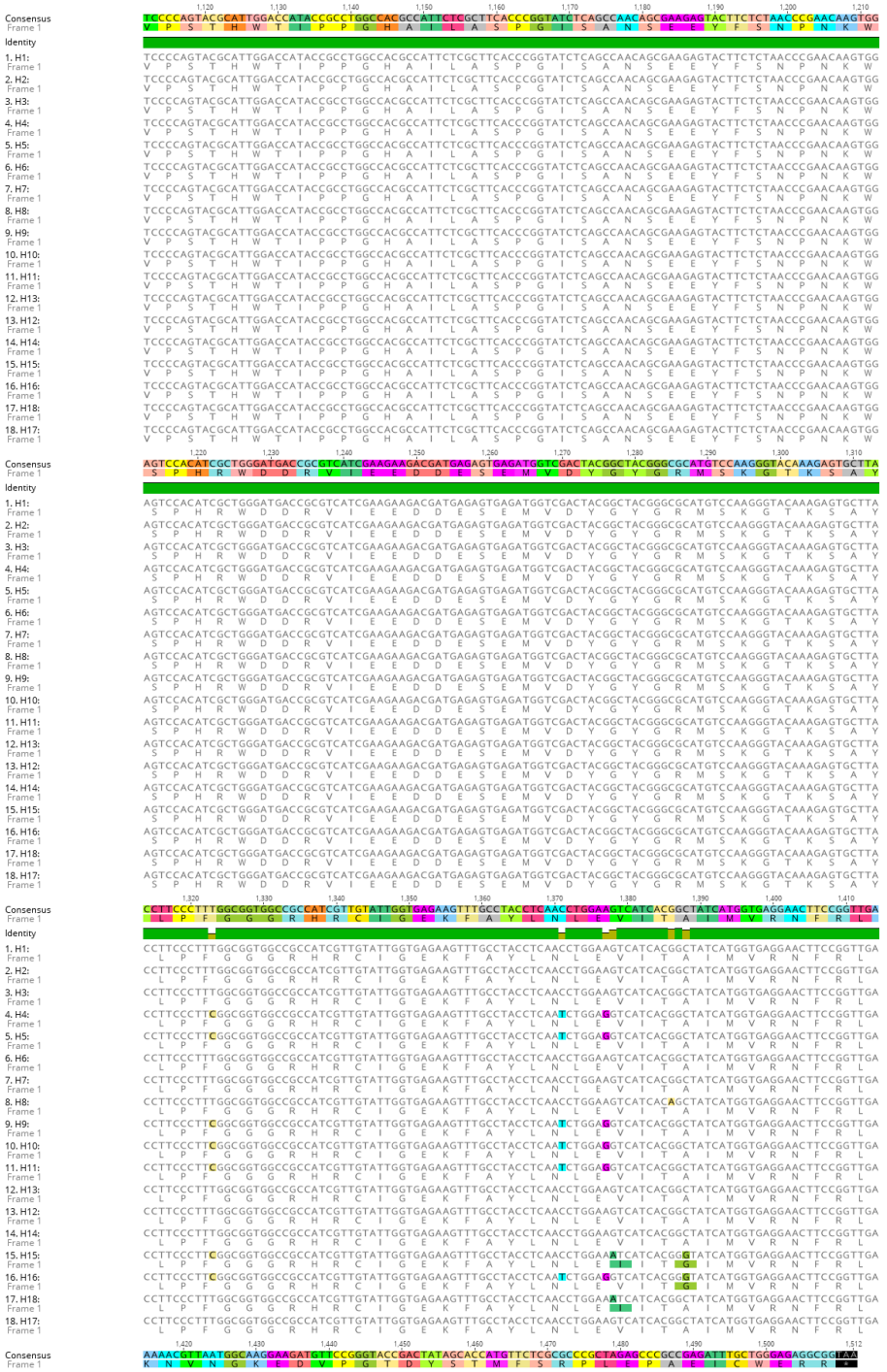
Consensus
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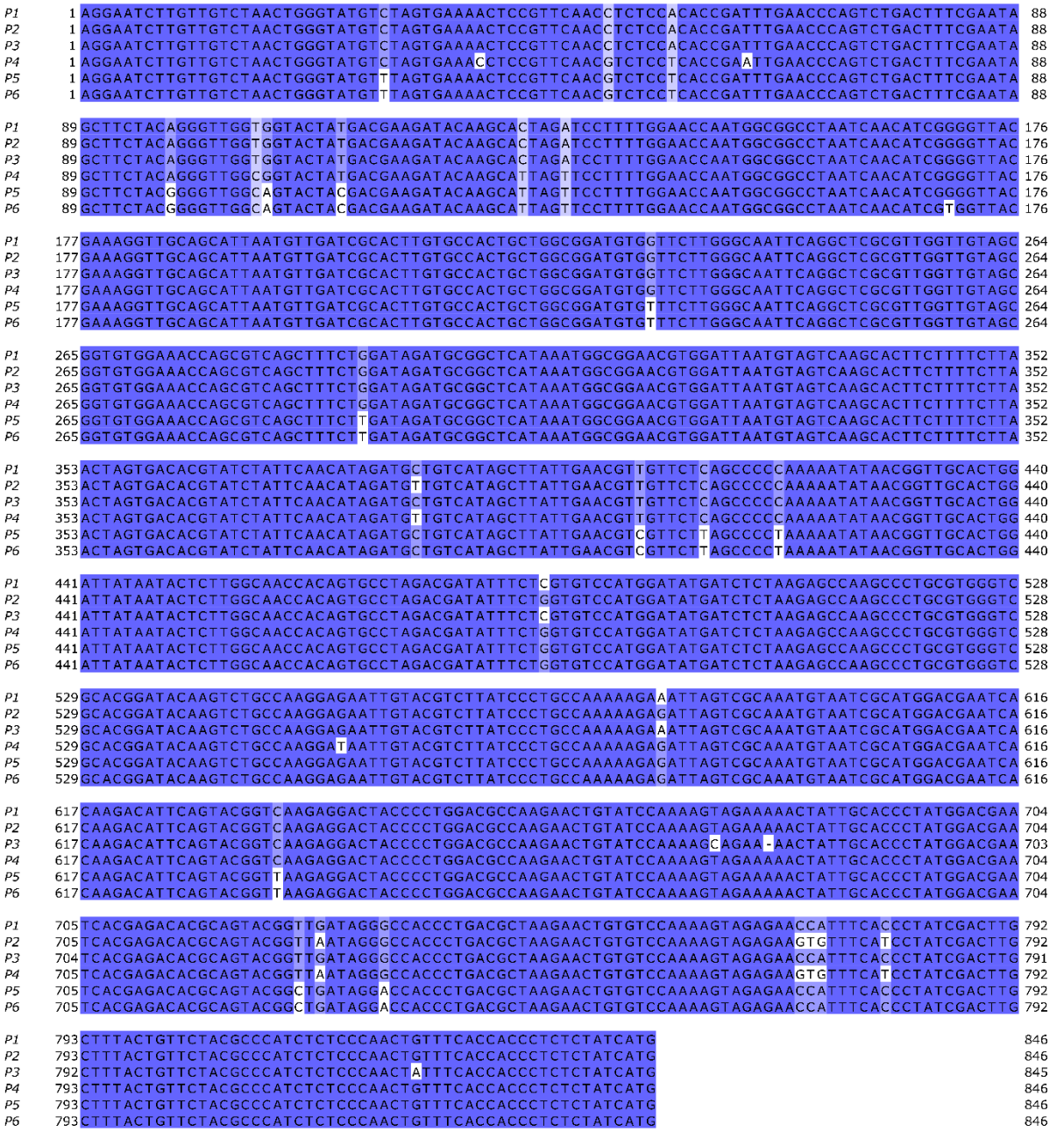
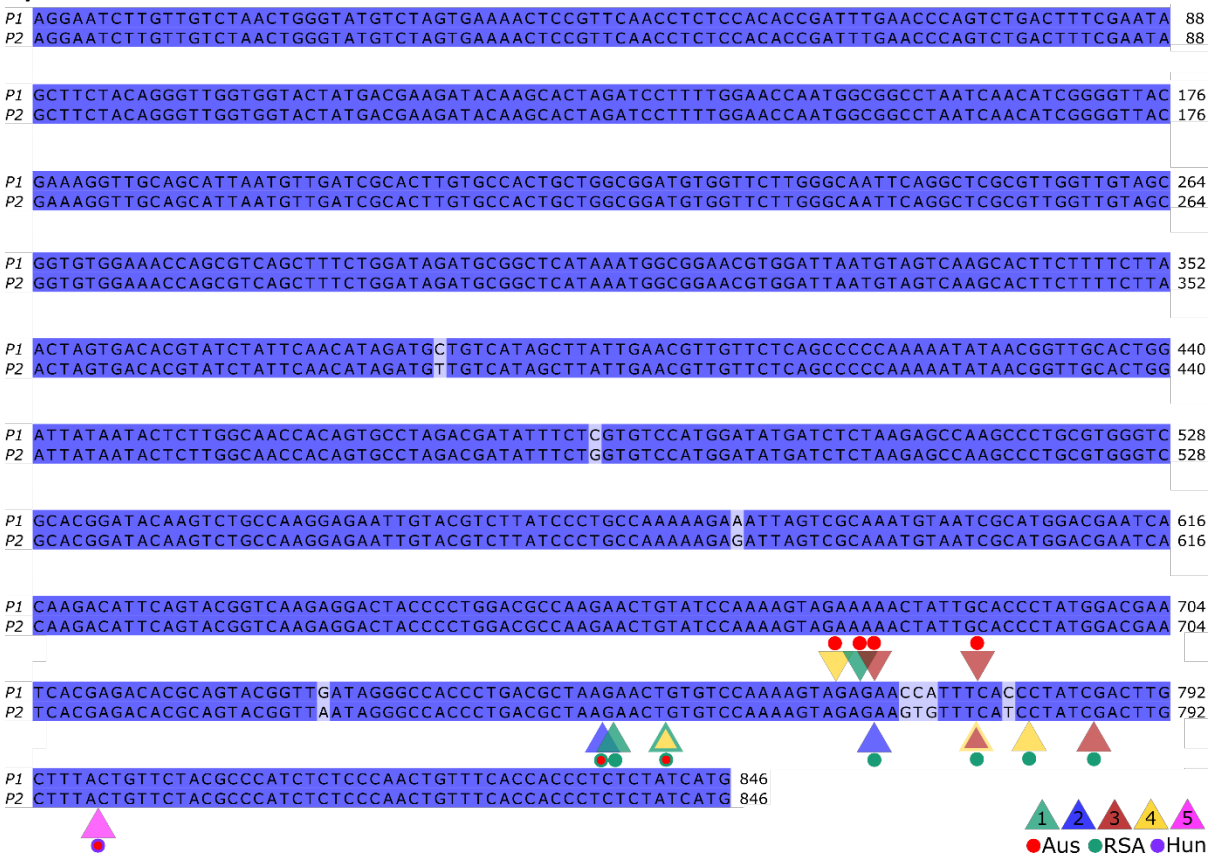
9. H9:
10. H10:
11. H11:
12. H13:
13. H12:
14. H14:
15. H15:
16. H16:
17. H18:
18. H17:

Supplemental File 2. Sequence alignments of six haplotypes of an 846 bp region of the *Ptm Cyp51A* promoter, excluding transposon insertions. P1 was detected in Australian isolates; P2 in RSA, Australian and Hungarian isolates; P3 in Hungarian and Turkish isolates; and P4, P5 and P6 in Turkish isolates. The alignment was created using ClustalW in Geneious v R9 (https://www.geneious.com).

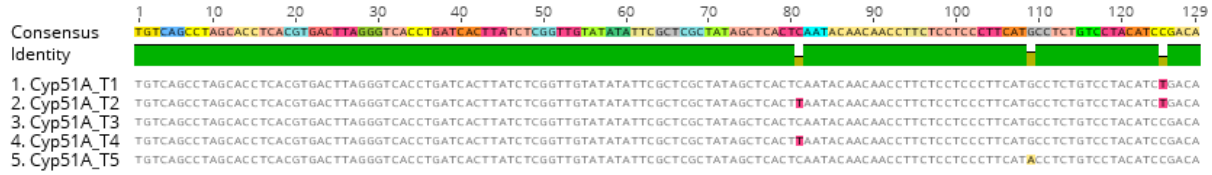


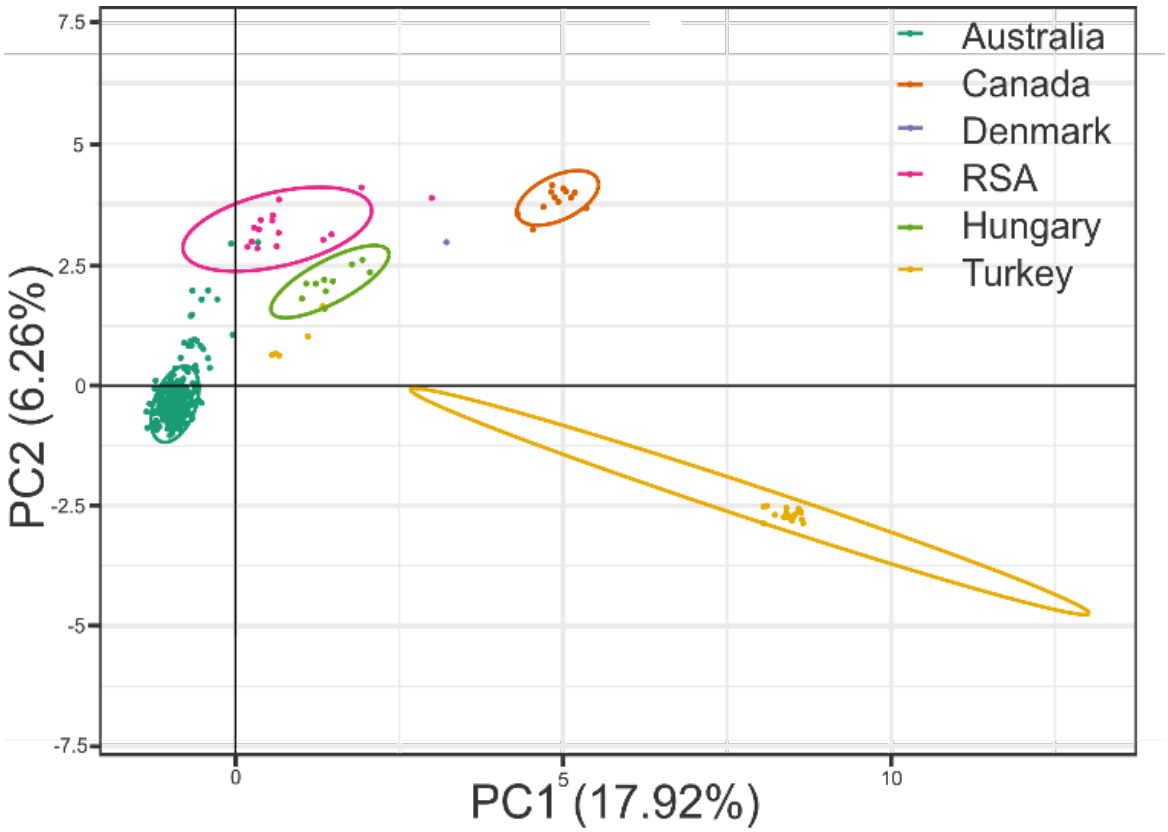
Supplemental File 3. A) Sequence alignments of the *Ptm Cyp51A* promoter P1 and P2 haplotypes with the insertion sites of five *Ty1-Copia* LTR retrotransposon-like alleles. Dual coloured triangles or circles indicate insertion of two alleles at one site or presence in isolates from two countries, respectively. B) Alignment of the retrotransposon-like sequences with SNP differences highlighted. Alignments were created using ClustalW in Geneious v R9 (https://www.geneious.com).

A)

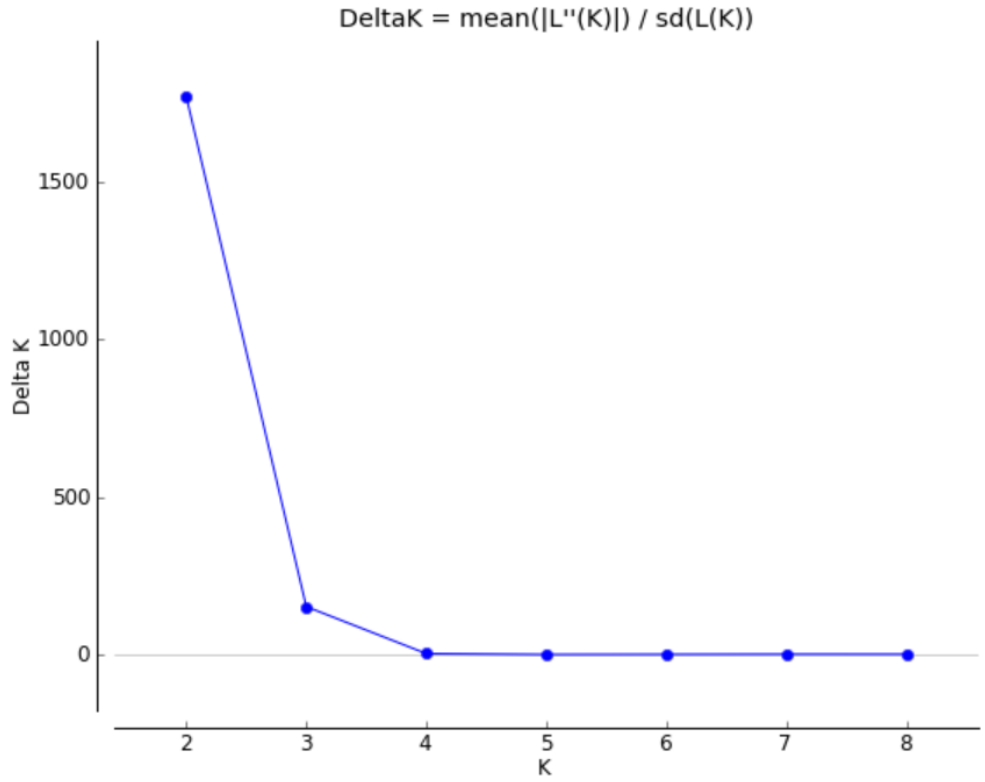


B)

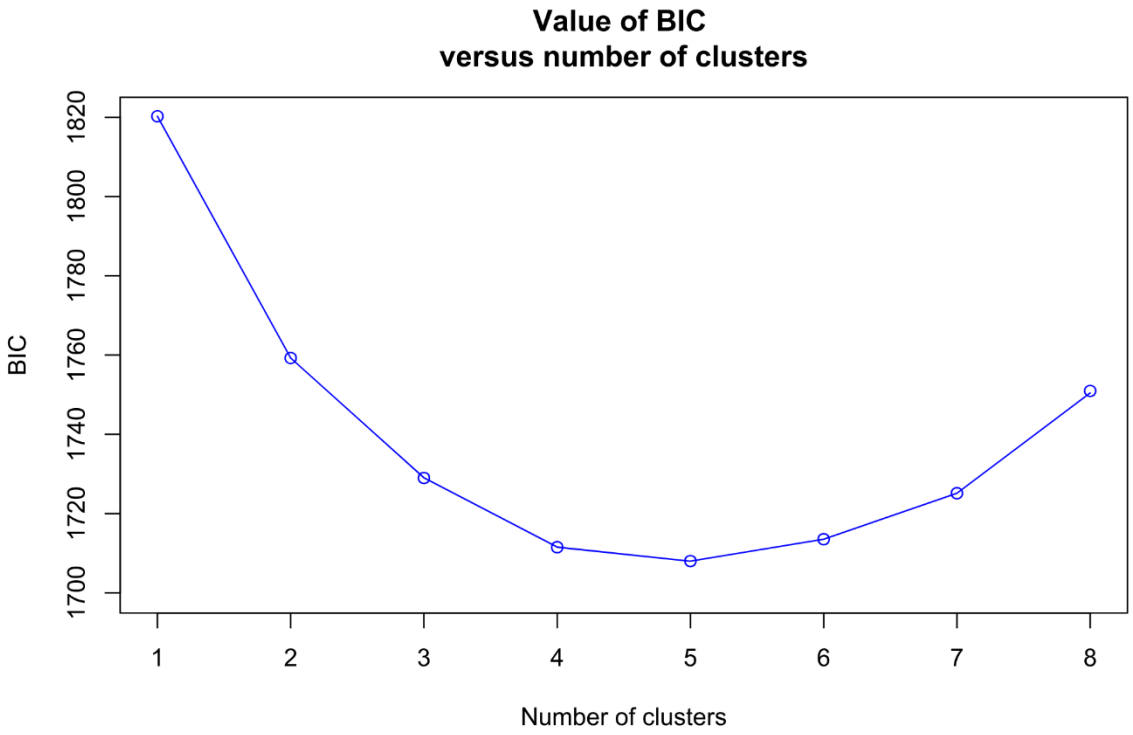




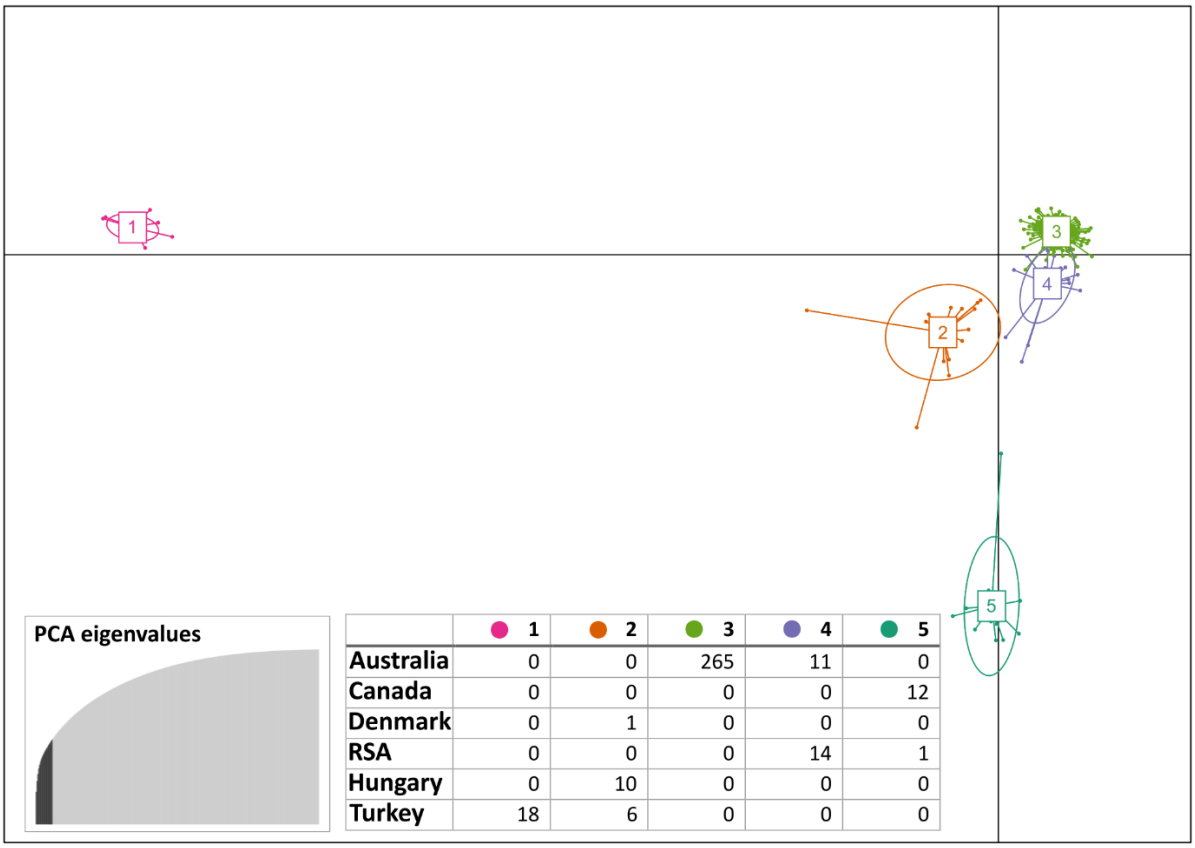
Supplemental Figure 9. Principal components analysis of *Ptm* isolates. The first two principal components are presented, with principal component 1 (PC1) explaining 17.9% of the variation and principal component 2 (PC2) explaining 6.2%. Ninety-five percent confidence ellipses broadly correspond with the origin of isolates from Australia, Canada, Denmark, the RSA and Turkey.



Supplemental Figure 10. Structure Harvester cluster analysis of intercontinental *Ptm* isolates. The online software Structure Harvester (Earl and von Holdt 2012) was used to determine the most likely number of subpopulations based on Q-value outputs from STRUCTURE v 2.3.4 using the Evanno Δk method (Evanno, *et al*., 2005). The most optimal number of clusters inferred in this model is two, based on 338 *Ptm* genotypes and 1,233 SNP and SillicoDArT markers. The Evanno Δk method uses the greatest change in magnitude of the second-order rate of change in ln Pr(X|K) against successive K values to indicate the most optimal K value (Gilbert, 2016).

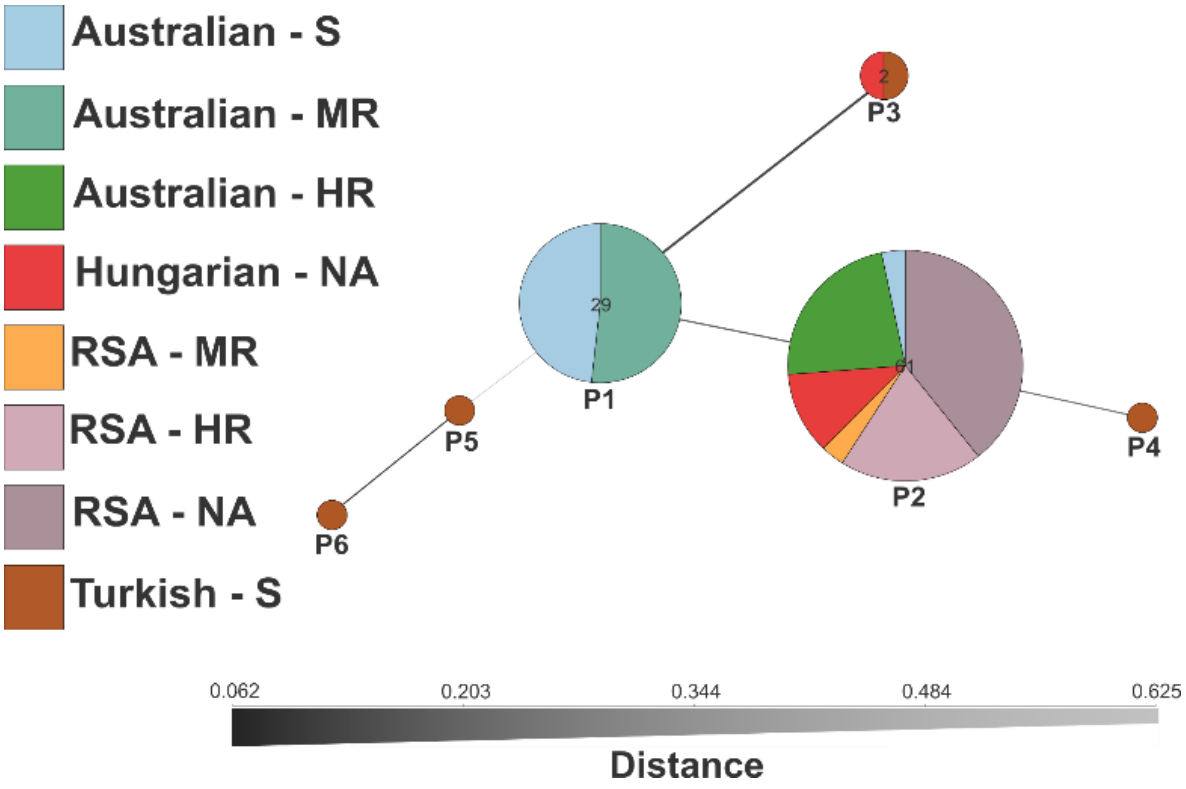


Supplemental Figure 11. Discriminant analysis of principal components (DAPC) cluster analysis of intercontinental *Ptm* isolates. DAPC (Jombart *et al*. 2010) was performed using 338 *Ptm* genotypes and a combined 1,233 SNP and SillicoDArT markers. In order to produce the line plot, successive runs of K-means are recorded with an increasing number of clusters (K), after transforming the isolate SNP data using a principal component analysis (PCA). For each cluster, a BIC (Bayesian information criterion) value was computed and the optimal K value inferred from the 'elbow' inflection, here depicted as five.



	1	2	3	4	5
Australia	0	0	265	11	0
Canada	0	0	0	0	12
Denmark	0	1	0	0	0
RSA	0	0	0	14	1
Hungary	0	10	0	0	0
Turkey	18	6	0	0	0

Supplemental Figure 12. Discriminant analysis of principal components of intercontinental *Ptm* isolates. Ninety-five percent confidence ellipses encircle each cluster. The inset table depicts isolate origins in the left hand column and rows with isolate groupings to each cluster.



Supplemental Figure 13. Minimum spanning network of the *Ptm Cyp51A* promoter region (without transposon insertions) describing international haplotype relationships. Each circle (node) represents a unique promoter variant and each node size is proportional to the number of sampled isolates. Isolate origins and fungicide phenotypes are presented as proportionate sectors of nodes with thicker lines between nodes representing higher genetic relatedness. HR, MR, S and NA indicate highly resistant, moderately resistant, sensitive isolate responses to tebuconazole or data not available, respectively.

Supplemental Table 1. Collection site metadata for *Ptm* isolates used for DArTseq.

Isolate ID	Collection date	Collector	Supplier	Host (cultivar)	Country	Region	GPS latitude	GPS longitude
19PTX020	15/07/2019	-	CCDM	*H. vulgare* (Bass)	Australia	WA	-33.7544	116.6806
86/16a	1/01/2017	Hugh Wallwork	AgVic	*H. vulgare* (Charger)	Australia	SA	-33.3999	138.1720
ptm19-086	5/07/2019	Angus Elder	AgVic	*H. vulgare* (Commander)	Australia	NSW	-29.1054	150.3089
ptm18-157	19/09/2018	Judy McIlroy	AgVic	*H. vulgare* (Dash)	Australia	Qld	-27.8515	152.3360
68/18	2018	Zack Zweck	SARDI	*H. vulgare* (Fleet)	Australia	SA	-36.0269	140.3418
19PTX085	14/08/2019	-	CCDM	*H. vulgare* (Flinders)	Australia	WA	-34.1354	118.9793
ptm19-198	28/09/2019	Grant Hollaway	AgVic	*H. vulgare* (Flinders)	Australia	Vic	-36.3974	145.5240
ptm19-203	2/10/2019	Judy	AgVic	*H. vulgare* (Flinders)	Australia	Qld	-27.5489	152.3272
19PTM054	22/07/2019	-	CCDM	*H. vulgare* (Granger)	Australia	WA	-33.6092	121.8993
ptm19-080	5/07/2019	Russell Wood	AgVic	*H. vulgare* (Grout)	Australia	Qld	-26.7502	150.6273
Ptm17-045	24/08/2017	Grant Hollaway	AgVic	*H. vulgare* (Hindmarsh)	Australia	Vic	-35.0549	142.9000
19PTM024	5/09/2019	-	CCDM	*H. vulgare* (La Trobe)	Australia	WA	-33.9630	118.7600
19PTM025	2/09/2019	-	CCDM	*H. vulgare* (La Trobe)	Australia	WA	-33.6847	122.2441
19PTX074	5/08/2019	-	CCDM	*H. vulgare* (La Trobe)	Australia	WA	-33.0734	121.7647

19PTX093	14/08/2019	-	CCDM	*H. vulgare* (La Trobe)	Australia	WA	-33.2088	121.2789
20PTM007	23/10/2020	Nola Dsouza	CCDM	*H. vulgare* (La Trobe)	Australia	WA	-31.9715	116.7805
87/16a	1/01/2017	Hugh Wallwork	AgVic	*H. vulgare* (Maltstar)	Australia	SA	-34.2128	138.0074
27A	14/10/2021	Kealan Hassett	CCDM	*H. vulgare* (Maximus Cl)	Australia	WA	-34.5630	117.8688
19PTX057	24/07/2019	-	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.0178	118.7162
19PTX065	30/07/2019	-	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-33.5644	118.2537
19PTX158	13/09/2019	-	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-32.1211	116.7701
19PTX190	1/10/2019	-	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-32.4934	117.4998
20P3001	23/09/2020	Kealan Hassett	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.5167	117.8905
20P3002	23/09/2020	Kealan Hassett	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.5167	117.8905
20P3003	23/09/2020	Kealan Hassett	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.5167	117.8905
20P3004	23/09/2020	Kealan Hassett	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.5167	117.8905
20PTM023	9/09/2020	Linda Thomson	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-33.9352	118.0055
20PTM040	12/10/2020	Linda Thomson	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-33.9622	118.4839
25E	14/10/2021	Kealan Hassett	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.5030	117.8688
61/18	2018	Hugh Wallwork	SARDI	*H. vulgare* (RGT Planet)	Australia	SA	-36.1051	140.3775

P2X1Y5	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X1Y8	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X1Y9	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X2Y1 (2)	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X2Y3	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X2Y7	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X2Y8	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X2Y9	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X3Y2	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X3Y6	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X3Y7	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X3Y8	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X4Y1	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X4Y5	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X4Y6	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X4Y9	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514

P2X5Y10	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X5Y4	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X5Y6 (2)	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X5Y7 (2)	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X5Y9 (2)	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X6Y4	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X6Y5	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X6Y6	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X6Y8	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P2X6Y9	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514
P3X1Y1	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X1Y10	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X1Y2	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X1Y3	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X1Y4	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X1Y7	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603

P3X2Y1	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X2Y10	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X2Y3	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X2Y4	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X2Y6	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X2Y9	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X3Y10	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X3Y4	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X3Y5	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X3Y6	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X3Y7	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X4Y1	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X4Y10	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X4Y2	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X4Y3	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X4Y4	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603

P3X4Y5	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X4Y6	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X4Y8	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X4Y9	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X5Y1	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X5Y10	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X5Y4	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X5Y5	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X5Y8	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X6Y1	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X6Y10 (2)	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X6Y2	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P3X6Y8	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P6X1Y1	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-33.6346	121.8919
P6X1Y10	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-33.6346	121.8919
P6X1Y4	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-33.6346	121.8919

P6X1Y8	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-33.6346	121.8919
P6X2Y8	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-33.6346	121.8919
P6X3Y10	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-33.6346	121.8919
P6X3Y4	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-33.6346	121.8919
P6X5Y8 (2)	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-33.6346	121.8919
P6X6Y4	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-33.6346	121.8919
P6X6Y5	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-33.6346	121.8919
19PTX060	29/07/2019	-	CCDM	*H. vulgare* (Rosalind)	Australia	WA	-34.6098	118.3738
19PTX081	5/08/2019	-	CCDM	*H. vulgare* (Rosalind)	Australia	WA	-34.4755	117.4766
29/18	2018	Hugh Wallwork	SARDI	*H. vulgare* (Rosalind)	Australia	SA	-33.8393	138.0106
ptm19-374	1/11/2019	Hugh Wallwork	AgVic	*H. vulgare* (Rosalind)	Australia	SA	-33.9397	136.2280
ptm19-376	1/11/2019	Mark McLean	AgVic	*H. vulgare* (Rosalind)	Australia	SA	-33.3999	138.1720
106/16a	1/01/2017	Hugh Wallwork	AgVic	*H. vulgare* (Scope)	Australia	Vic	-35.4868	142.9133
ptm19-375	1/11/2019	Mark McLean	AgVic	*H. vulgare* (Scope)	Australia	SA	-35.4029	139.5326
ptm19-377	1/11/2019	Mark McLean	AgVic	*H. vulgare* (Scope)	Australia	SA	-33.8393	138.0106
71/17	2017	Hugh Wallwork	SARDI	*H. vulgare* (SFR 85-009)	Australia	SA	-36.1435	140.3426

19C	14/10/2021	Kealan Hassett	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5165	118.0147
19D	14/10/2021	Kealan Hassett	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5165	118.0147
19E	14/10/2021	Kealan Hassett	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5165	118.0147
19PTM049	19/09/2019	-	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-31.7503	117.4935
19PTM098	4/09/2019	-	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-31.3927	117.3380
19PTM425	8/10/2019	-	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.8154	116.9542
19PTX069	30/07/2019	-	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-32.6121	116.7419
19PTX070	29/07/2019	-	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.7544	117.3057
19PTX082	1/08/2019	-	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.1788	118.1716
19PTX091	18/08/2019	-	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.1459	117.4059
19PTX147	9/09/2019	-	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-31.4359	117.2857
19PTX153	13/09/2019	-	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-31.4210	118.4775
19PTX168	19/09/2019	-	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-31.0445	117.0606
19PTX172	19/09/2019	-	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-31.2487	116.9059
22/17	2017	-	SARDI	*H. vulgare* (Spartacus Cl)	Australia	SA	-33.1327	136.4113
24A	14/10/2021	Kealan Hassett	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5165	118.0147

24B	15/10/2021	Kealan Hassett	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5165	118.0147
25/17	2017	Hugh Wallwork	SARDI	*H. vulgare* (Spartacus Cl)	Australia	SA	-34.3338	140.8165
39/18	2018	Martyn Chandler	SARDI	*H. vulgare* (Spartacus Cl)	Australia	SA	-34.4710	140.5752
48/18	2018	Blake Gontar	SARDI	*H. vulgare* (Spartacus Cl)	Australia	SA	-34.3351	135.8919
ptm19-378	1/11/2019	Mark McLean	AgVic	*H. vulgare* (Spartacus Cl)	Australia	SA	-33.8393	138.0106
S2X1Y2	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X1Y3	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X1Y3 (2)	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X1Y4	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X1Y5 (2)	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X1Y7	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X2Y1	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X2Y10	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X2Y2	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X2Y4	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X2Y5	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514

S2X2Y7_2	26/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X2Y9	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X3Y1	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X3Y10	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X3Y3	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X3Y8	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X4Y1	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X4Y3	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X4Y6 (2)	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X5Y1	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X5Y10 (2)	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X5Y2	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X5Y3	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X5Y9	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X6Y3	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S2X6Y5	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514

S2X6Y8 (2)	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514
S3X1Y1	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X1Y2	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X1Y4	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X1Y7	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X2Y1	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X3Y2	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X3Y4	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X3Y5	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X3Y5 (2)	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X3Y6	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X3Y7	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X3Y7 (2)	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X4Y10	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X4Y3	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X4Y4	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145

S3X4Y5	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X4Y9 (2)	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X5Y10 (2)	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X5Y2 (2)	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X5Y5	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X5Y6 (2)	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X5Y9	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X6Y1	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X6Y5	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X6Y6	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X6Y7	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S3X6Y7 (2)	24/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-34.5150	118.0145
S6X1Y10	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X1Y2	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X1Y5	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X1Y6	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441

S6X1Y8	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X2Y1	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X2Y10	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X2Y4	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X2Y5	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X2Y7	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X2Y8 (2)	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X3Y1	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X3Y10	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X3Y5	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X3Y6	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X3Y7	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X3Y8	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X4Y1	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X4Y4	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X4Y5	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441

S6X4Y8	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X4Y9	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X5Y1	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X5Y10	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X5Y3	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X5Y4	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X5Y5	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X5Y6	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X5Y7	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X5Y8	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X6Y1	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X6Y3	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X6Y6	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X6Y7	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
S6X6Y8	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
T2	28/08/2020	Kealan Hassett	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-31.9529	116.8697

T3	28/08/2020	Kealan Hassett	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-31.9529	116.8697
TX1Y1	28/08/2020	Kealan Hassett	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-31.9529	116.8697
59/17	2017	Hugh Wallwork	SARDI	*H. vulgare* (Spartacus Cl)	Australia	SA	-33.9397	136.2280
09-038	2010	Mark McLean	AgVic	*H. vulgare*	Australia	Vic	-29.3053	149.7773
16D	15/10/2021	Kealan Hassett	CCDM	*H. vulgare*	Australia	WA	-33.7031	117.1147
17C	13/10/2021	Kealan Hassett	CCDM	*H. vulgare*	Australia	WA	-34.3764	117.5501
17D	13/10/2021	Kealan Hassett	CCDM	*H. vulgare*	Australia	WA	-34.3764	117.5501
18B	15/10/2021	Kealan Hassett	CCDM	*H. vulgare*	Australia	WA	-33.7228	117.1081
18C	15/10/2021	Kealan Hassett	CCDM	*H. vulgare*	Australia	WA	-33.7228	117.1081
18D	15/10/2021	Kealan Hassett	CCDM	*H. vulgare*	Australia	WA	-33.7228	117.1081
18E	15/10/2021	Kealan Hassett	CCDM	*H. vulgare*	Australia	WA	-33.7228	117.1081
18F	15/10/2021	Kealan Hassett	CCDM	*H. vulgare*	Australia	WA	-33.7228	117.1081
34/17	2017	Hugh Wallwork	SARDI	*H. vulgare*	Australia	SA	-33.8944	136.5832
41/17	2017	Hugh Wallwork	SARDI	*H. vulgare*	Australia	SA	-35.2470	140.9523
Hal_004	13/09/2012	Simon Elwood	CCDM	*H. vulgare*	Australia	WA	-34.5555	117.6495
M3	3/10/2009	Simon Elwood	CCDM	*H. vulgare*	Australia	WA	-31.7264	116.7044
M4	3/10/2009	Simon Elwood	CCDM	*H. vulgare*	Australia	WA	-31.7264	116.7044
Ptm17-038	19/09/2017	Mark McLean	AgVic	*H. vulgare*	Australia	Vic	-36.7129	142.8686
Ptm17-044	24/08/2017	Grant Hollaway	AgVic	*H. vulgare*	Australia	Vic	-35.6880	142.0908
Ptm17-047	19/09/2017	Mark McLean	AgVic	*H. vulgare*	Australia	Vic	-36.2441	142.5560
Ptm17-054	17/10/2017	Mark McLean	AgVic	*H. vulgare*	Australia	Vic	-37.9465	143.2643
Ptm17-056	25/09/2017	Mark McLean	AgVic	*H. vulgare*	Australia	Vic	-36.7833	142.3416
Ptm17-057	19/09/2017	Mark McLean	AgVic	*H. vulgare*	Australia	Vic	-36.2366	142.6873
Ptm17-059	29/09/2017	Mark McLean	AgVic	*H. vulgare*	Australia	Vic	-35.7178	143.0859
Ptm17-075	17/10/2017	Mark McLean	AgVic	*H. vulgare*	Australia	Vic	-38.0838	143.9379
ptm18-013	3/09/2018	Mark McLean	AgVic	*H. vulgare*	Australia	Vic	-36.9903	142.8740
ptm18-015	2/08/2018	Mark McLean	AgVic	*H. vulgare*	Australia	Vic	-36.4793	142.3634
ptm18-019	3/09/2018	Mark McLean	AgVic	*H. vulgare*	Australia	Vic	-35.9877	142.4162

ptm18-024	14/09/2018	Mark McLean	AgVic	*H. vulgare*	Australia	Vic	-36.5057	142.1052
ptm18-025	14/09/2018	Mark McLean	AgVic	*H. vulgare*	Australia	Vic	-36.6990	142.1677
ptm18-026	1/10/2018	Mark McLean	AgVic	*H. vulgare*	Australia	Vic	-36.6202	142.4710
ptm18-136	14/09/2018	Mark McLean	AgVic	*H. vulgare*	Australia	Vic	-36.6619	141.2751
ptm18-156	29/08/2018	Leigh Norton	AgVic	*H. vulgare*	Australia	NSW	-29.6348	150.8502
ptm18-158	28/09/2018	Leigh Norton	AgVic	*H. vulgare*	Australia	NSW	-28.8959	150.0780
ptm18-160	19/09/2018	Judy McIlroy	AgVic	*H. vulgare*	Australia	Qld	-27.8515	152.3360
ptm19-047	28/08/2019	Grant Hollaway	AgVic	*H. vulgare*	Australia	Vic	-35.4333	142.2232
ptm19-055	5/09/2019	Mark Mclean	AgVic	*H. vulgare*	Australia	Vic	-36.4079	142.0176
ptm19-057	5/09/2019	Mark Mclean	AgVic	*H. vulgare*	Australia	Vic	-35.1940	141.3689
ptm19-059	30/08/2019	Mark Mclean	AgVic	*H. vulgare*	Australia	Vic	-35.2935	143.3422
ptm19-060	29/08/2019	Mark Mclean	AgVic	*H. vulgare*	Australia	Vic	-36.0691	142.4849
ptm19-061	5/09/2019	Mark Mclean	AgVic	*H. vulgare*	Australia	Vic	-35.2363	140.9805
ptm19-065	30/08/2019	Mark Mclean	AgVic	*H. vulgare*	Australia	Vic	-34.3481	142.1948
ptm19-069	5/09/2019	Mark Mclean	AgVic	*H. vulgare*	Australia	Vic	-35.9517	141.9797
ptm19-197	22/10/2019	Grant Hollaway	AgVic	*H. vulgare*	Australia	Vic	-36.0309	146.0080
ptm19-200	4/10/2019	Hari Dadu	AgVic	*H. vulgare*	Australia	Vic	-35.8245	142.1769
ptm19-202	25/09/2019	Janet	AgVic	*H. vulgare*	Australia	Qld	-26.6105	153.0232
ptm19-207	18/10/2019	Mark McLean	AgVic	*H. vulgare*	Australia	Vic	-38.1079	144.1466
ptm19-420	27/02/2020	Blue	AgVic	*H. vulgare*	Australia	WA	-33.7341	122.7850
SG1 #	1996	Ian Goss	CCDM	*H. vulgare*	Australia	WA	-30.4440	115.6148
U7	2012	-	CCDM	*H. vulgare*	Australia	WA	-34.2342	118.2913
20PTM048	16/10/2020	Linda Thomson	CCDM	*H. vulgare*	Australia	WA	-33.8612	121.8921
143/16a	1/01/2017	Hugh Wallwork	AgVic	*H. vulgare* (Westminster)	Australia	SA	-37.1193	140.1892
72/17	2017	Hugh Wallwork	SARDI	*H. vulgare* (Westminster)	Australia	SA	-37.1192	140.1945
Ptm17-052	18/10/2017	Mark McLean	AgVic	*H. vulgare* (Westminster)	Australia	Vic	-37.6393	143.0777
AB57	2010	-	Agri-Food Canada	*H. vulgare*	Canada	Alberta (Rimbey)	52.6380	-114.2458

AB68	2010	-	Agri-Food Canada	*H. vulgare*	Canada	Alberta (Lousana)	52.1138	-113.1934
AB82	2011	-	Agri-Food Canada	*H. vulgare*	Canada	Alberta (Rosedale valley)	52.4685	-113.7307
OAC21	2003	-	Agri-Food Canada	*H. vulgare (6 row barley)*	Canada	Saskatchewan (Melfort)	52.8608	-104.6143
CDCTrey	2005	-	Agri-Food Canada	*H. vulgare (2 row barley)*	Canada	Manitoba (Brandon)	49.8485	-99.9501
Dillon	2004	-	Agri-Food Canada	*H. vulgare (6 row barley)*	Canada	Alberta (Blackfalds)	52.3809	-113.7954
LO262	2005	-	Agri-Food Canada	*H. vulgare*	Canada	Alberta (Central)	53.9333	-116.5765
MBV25	2012	-	Agri-Food Canada	*H. vulgare*	Canada	Manitoba (Roblin)	51.2282	-101.3523
MBV37	2012	-	Agri-Food Canada	*H. vulgare*	Canada	Manitoba (Portage la Prairie)	49.9723	-98.2903
SK60	2010	-	Agri-Food Canada	*H. vulgare*	Canada	Saskatchewan (Stony Beach)	50.4689	-105.1681
TR253	2005	-	Agri-Food Canada	*H. vulgare*	Canada	Manitoba (Brandon)	49.8485	-99.9501
WRS857	1973	-	Agri-Food Canada	*H. vulgare (Oakbank)*	Canada	Manitoba (Winnipeg)	49.8075	-97.1366
CBS 228.76	1970's	V. Smedegaard-Petersen	University of Southern Queensland	*H. vulgare*	Denmark	-	55.4259	9.7411
H-160	2007	Bakonyi József	Agrártudományi Kutatóközpont	*H. vulgare*	Hungary	Kiszombor-Makó	46.1810	20.4264
H-801	7/06/2017	Bakonyi József	Agrártudományi Kutatóközpont	*H. vulgare (Antonella)*	Hungary	Karcag	47.3100	20.8894

H-812	7/06/2017	Bakonyi József	Agrártudományi Kutatóközpont	*H. vulgare (GK Judy)*	Hungary	Karcag	47.3100	20.8894
H-896	2018	Bakonyi József	Agrártudományi Kutatóközpont	*H. vulgare*	Hungary	Karcag (Univ. Debrecen, AGTC KIT Res. Inst. Karcag, exp. field) (HU)	47.3100	20.8894
H-917	6/06/2018	Bakonyi József	Agrártudományi Kutatóközpont	*H. vulgare (KG Konta)*	Hungary	Kompolt	47.7189	20.2375
H-918	6/06/2018	Bakonyi József	Agrártudományi Kutatóközpont	*H. vulgare (KG Konta)*	Hungary	Kompolt	47.7189	20.2375
H-933	6/06/2018	Bakonyi József	Agrártudományi Kutatóközpont	*H. vulgare (Patina)*	Hungary	Kompolt	47.7189	20.2375
H-947	6/06/2018	Bakonyi József	Agrártudományi Kutatóközpont	*H. vulgare (KWS Meridian)*	Hungary	Kompolt	47.7189	20.2375
H-953	6/06/2018	Bakonyi József	Agrártudományi Kutatóközpont	*H. vulgare (Kompolt)*	Hungary	Kompolt	47.7189	20.2375
H-117.1/1	1992	Jozsef Bakonyi	Agrártudományi Kutatóközpont	*H. vulgare*	Hungary	Tapolca	46.8954	17.4453
CG16020	2016	Noel Knight	University of Southern Queensland	*H. vulgare*	RSA	Southern Cape (Dunghye Park)	-34.3299	19.5268
CG16022	2016	Noel Knight	University of Southern Queensland	*H. vulgare*	RSA	Southern Cape (Dunghye Park)	-34.3299	19.5268

CG16025	2016	Noel Knight	University of Southern Queensland	*H. vulgare*	RSA	Southern Cape (Dunghye Park)	-34.3299	19.5268
CG16026	2016	Noel Knight	University of Southern Queensland	*H. vulgare*	RSA	Southern Cape (Dunghye Park)	-34.3299	19.5268
CG16027	2016	Noel Knight	University of Southern Queensland	*H. vulgare*	RSA	Southern Cape (Dunghye Park)	-34.3299	19.5268
CG16033	2016	Noel Knight	University of Southern Queensland	*H. vulgare*	RSA	Southern Cape (Dunghye Park)	-34.3299	19.5268
CG16039	2016	Noel Knight	University of Southern Queensland	*H. vulgare*	RSA	Southern Cape (Dunghye Park)	-34.3299	19.5268
CG16045	2016	Noel Knight	University of Southern Queensland	*H. vulgare*	RSA	Southern Cape (Dunghye Park)	-34.3299	19.5268
CG16046	2016	Noel Knight	University of Southern Queensland	*H. vulgare*	RSA	Southern Cape (Dunghye Park)	-34.3299	19.5268
CG16059	2016	Noel Knight	University of Southern Queensland	*H. vulgare*	RSA	Southern Cape (Greyton)	-34.0534	19.6104
CG16060	2016	Noel Knight	University of Southern Queensland	*H. vulgare*	RSA	Southern Cape (Napier)	-34.4754	19.8961
CG16066	2016	Noel Knight	University of Southern Queensland	*H. vulgare*	RSA	Southern Cape (Napier)	-34.4754	19.8961
CG16069	2016	Noel Knight	University of Southern Queensland	*H. vulgare*	RSA	Southern Cape (Protem)	-34.2667	20.0833

CG16071	2016	Noel Knight	University of Southern Queensland	*H. vulgare*	RSA	Southern Cape (Protem)	-34.2667	20.0833
CG16074	2016	Noel Knight	University of Southern Queensland	*H. vulgare*	RSA	Southern Cape (Klipdale)	-34.3041	19.9720
CG16080	2016	Noel Knight	University of Southern Queensland	*H. vulgare*	RSA	Southern Cape (Klipdale)	-34.3041	19.9720
Gps116	2017	Aziz Karakaya	Ankara University	*H. vulgare*	Turkey	Central (Konyaz, Bozkır)	37.1889	32.2454
Gps99	2017	Aziz Karakaya	Ankara University	*H. vulgare*	Turkey	Central (Konya, Yunak)	38.8135	31.7357
Gps265	2017	Aziz Karakaya	Ankara University	*H. vulgare*	Turkey	Central (Ankara, Şereflikoçhisar)	38.9379	33.5392
13-130	2017	Aziz Karakaya	Ankara University	*H. vulgare*	Turkey	Eastern (Şanlıurfa, Ceylanpınar)	36.9092	39.7343
Gps76	2017	Aziz Karakaya	Ankara University	*H. vulgare*	Turkey	Central (Ankara, Kalecik)	40.1065	33.4114
Gps70	2017	Aziz Karakaya	Ankara University	*H. vulgare*	Turkey	Central (Kırşehir, Kaman)	39.3589	33.7234
13-163	2017	Aziz Karakaya	Ankara University	*H. vulgare*	Turkey	Eastern (Diyarbakır, Central District)	38.1066	40.5427
Gps56	2017	Aziz Karakaya	Ankara University	*H. vulgare*	Turkey	Central (Yozgat, Boğazlıyan)	39.1937	35.2468
Gps19	2017	Aziz Karakaya	Ankara University	*H. vulgare*	Turkey	Central (Sivas, Central District)	39.7505	37.0150
Gps27	2017	Aziz Karakaya	Ankara University	*H. vulgare*	Turkey	Central (Sivas Şarkışla)	39.3502	36.4078
Gps8	2017	Aziz Karakaya	Ankara University	*H. vulgare*	Turkey	Central (Kırıkkale, Delice)	39.9437	34.0326
Gps79	2017	Aziz Karakaya	Ankara University	*H. vulgare*	Turkey	Central (Çankırı, Central District)	40.5369	33.5884

13-181	2017	Aziz Karakaya	Ankara University	*H. vulgare*	Turkey	Eastern (Kahramanmaraş, Pazarcık)	37.5753	36.9228
Edirne	2017	Aziz Karakaya	Ankara University	*H. vulgare*	Turkey	Erdine	41.6771	26.5557
13-116	2017	Aziz Karakaya	Ankara University	*H. vulgare*	Turkey	Central (Niğde, Ulukışla)	37.6045	34.5971
Gps276	2017	Aziz Karakaya	Ankara University	*H. bulbosum*	Turkey	Eastern (Kilis)	36.7165	37.1147
13-142	2017	Aziz Karakaya	Ankara University	*H. vulgare*	Turkey	Eastern (Mardin , Ömerli)	37.4031	40.9548
Afyon	2017	Aziz Karakaya	Ankara University	*H. vulgare*	Turkey	Central (Afyon)	38.7569	30.5387
Gps43	2017	Aziz Karakaya	Ankara University	*H. vulgare*	Turkey	Central (Kayseri, Bünyan)	38.8551	35.8521
Gps81	2017	Aziz Karakaya	Ankara University	*H. vulgare*	Turkey	Central (Çankırı, Ilgaz)	40.9252	33.6272
Gps125	2017	Aziz Karakaya	Ankara University	*H. vulgare*	Turkey	Central (Konya, Karatay)	37.8232	32.9888
Gps158	2017	Aziz Karakaya	Ankara University	*H. vulgare*	Turkey	Central (Eskişehir, Odunpazarı)	39.7667	30.5256
A9	2017	Aziz Karakaya	Ankara University	*H. vulgare*	Turkey	Central (Ankara, Nallıhan)	40.1889	31.3506
Gps187	2017	Aziz Karakaya	Ankara University	*H. vulgare*	Turkey	Central (Eskişehir, Beylikova)	39.6894	31.2058
S6X3Y8 (2)	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-33.6847	122.2441
P6X4Y7	7/10/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-33.6346	121.8919
P3X6Y6	23/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-34.4795	117.8603
P2X6Y7 (2)	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-30.8137	116.1514

19PTX180	19/09/2019	-	CCDM	*H. vulgare* (RGT Planet)	Australia	WA	-31.8454	117.9409
19PTX164	18/09/2019	-	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-31.2089	116.5835
S2X1Y9 (2)	25/09/2020	K. Hassett and J. M. Gonzalez	CCDM	*H. vulgare* (Spartacus Cl)	Australia	WA	-30.8137	116.1514

Supplemental Table 2. Collection site data for *Ptm* isolates used for *Cyp51A* gene and promoter DNA sequencing only.

Isolate ID	Collection date	Collector	Supplier	Cultivar	Country	Region (locality)	GPS latitude	GPS longitude
PTM15#1	2007	-	USQ	-	RSA	Southern Cape (Waenshuiskrans)	-34.6747	20.2238
PTM22	2007	-	USQ	-	RSA	Southern Cape (Waenshuiskrans)	-34.6747	20.2238
PTM21	2007	-	USQ	-	RSA	Southern Cape (Waenshuiskrans)	-34.6747	20.2238
PTM27#1	2007	-	USQ	-	RSA	Southern Cape (Waenshuiskrans)	-34.6747	20.2238
PTM23	2007	-	USQ	-	RSA	Southern Cape (Waenshuiskrans)	-34.6747	20.2238
PTM26	2007	-	USQ	-	RSA	Southern Cape (Waenshuiskrans)	-34.6747	20.2238
PTM71	2007	-	USQ	-	RSA	Southern Cape (Waenshuiskrans)	-34.6747	20.2238
PTM73	2007	-	USQ	-	RSA	Southern Cape (Waenshuiskrans)	-34.6747	20.2238
PTM14	2007	-	USQ	-	RSA	Southern Cape (Waenshuiskrans)	-34.6747	20.2238
PTM18	2007	-	USQ	-	RSA	Southern Cape (Waenshuiskrans)	-34.6747	20.2238
PTM55#2	2007	-	USQ	-	RSA	Southern Cape (Waenshuiskrans)	-34.6747	20.2238
PTM56#2	2007	-	USQ	-	RSA	Southern Cape (Waenshuiskrans)	-34.6747	20.2238
PTM57#1	2007	-	USQ	-	RSA	Southern Cape (Waenshuiskrans)	-34.6747	20.2238
PTM58#2	2007	-	USQ	-	RSA	Southern Cape (Waenshuiskrans)	-34.6747	20.2238
PTM19	2007	-	USQ	-	RSA	Southern Cape (Waenshuiskrans)	-34.6747	20.2238
PTM62#2	2007	-	USQ	-	RSA	Southern Cape (Waenshuiskrans)	-34.6747	20.2238
PTM64#1	2007	-	USQ	-	RSA	Southern Cape (Waenshuiskrans)	-34.6747	20.2238
PTM32	2007	-	USQ	-	RSA	Southern Cape (Riviersonderend)	-34.1405	19.9172
PTM67#2	2007	-	USQ	-	RSA	Southern Cape (Waenshuiskrans)	-34.6747	20.2238
PTM68#1	2007	-	USQ	-	RSA	Southern Cape (Waenshuiskrans)	-34.6747	20.2238
PTM69#2	2007	-	USQ	-	RSA	Southern Cape (Waenshuiskrans)	-34.6747	20.2238
PTM38	2007	-	USQ	-	RSA	Southern Cape (Swellendam)	-34.0256	20.4487
PTM24	2007	-	USQ	-	RSA	Southern Cape (Waenshuiskrans)	-34.6747	20.2238
PTM25	2007	-	USQ	-	RSA	Southern Cape (Waenshuiskrans)	-34.6747	20.2238
H-1016	5/06/2018	B. József	B. József	Siberia	Hungary	Martonvásár (Budapest)	47.3228	18.7993
18FRG195	2018	D. Cameron	CCDM	RGT Planet	Australia	WA (Pithara)	-30.4111	116.7045
17FRG089	13/09/2017	Kith Jayasena	CCDM	Oxford	Australia	WA (Takaralup)	-34.5785	118.1380

Supplemental Table 3. Analysis of molecular variance *of Pyrenophora teres* f. *maculata* isolates from Australia, Hungary, RSA, Turkey and Canada. Variance among regions was not calculated for the RSA since the number of samples per region were insufficient and a single isolate from Hungary was excluded.

Comparison	Degrees of freedom	Variation (%)	*p* value	Sum of squares	Mean Square
Australia, Hungary, RSA, Turkey and Canada					
Among countries	4	52.99	0.0001[a]	23146.65	4629.33
Within countries	332	47.01	0.0001[a]	59823.13	180.19
Australia					
Among regions	3	2.077	0.7732	833.04	277.67
Within regions	272	97.92	0.0001[a]	44208.17	162.53
Turkey					
Among regions	1	67.17	0.0001[a]	2256.56	2256.56
Within regions	21	32.83	0.0001[a]	2785.55	132.65
Canada					
Among regions	1	-0.27	0.4496	126.8	126.8
Within regions	8	100.3	0.0001[a]	1028	128.5

[a] Significant at $P < 0.01$.

Supplemental Table 4. Pairwise AMOVA genetic differentiation PhiPT values among *Ptm* isolate collections. The values are based on 999 permutations, with all values significantly greater than 0, $p < 0.0001$.

Origin	Australia	Canada	RSA	Hungary	Turkey
Australia	-				
Canada	0.576	-			
RSA	0.344	0.505	-		
Hungary	0.412	0.539	0.431	-	
Turkey	0.547	0.430	0.472	0.444	-

Supplemental Table 5. Overview of Cyp51A promoter Ty1-Copia LTR retrotransposon insertion sites and tebuconazole resistance phenotypes for 95 Ptm isolates. HR, MR, and S indicate highly resistant, moderately resistant, and sensitive, respectively. A dash indicates no promoter insertion and NA no live cultures were available for tebuconazole resistance tests. Cyp51A gene and promoter haplotype naming conventions are provided in Supplemental Table 7.

Isolate	Origin and tebuconazole resistance phenotype	*Cyp51A* haplotype	Promoter haplotype	*Cyp51A* 489 codon	Transposon insertion position from *Cyp51A* start codon	Maximum tebuconazole concentration with visual growth (µg ml^{-1})	LTR target site duplication sequence	Transposon allele number
19PTX147~	Australia - HR	H4	P2:95:2	TTG	-95	10	GCTAA	2
P2X2Y8~	Australia - HR	H4	P2:90:1	TTG	-90	10	GAACT	1
P2X4Y9~	Australia - HR	H4	P2:90:1	TTG	-90	10	GAACT	1
P2X5Y7(2)~	Australia - HR	H4	P2:90:1	TTG	-90	10	GAACT	1
P2X6Y5~	Australia - HR	H4	P2:90:1	TTG	-90	10	GAACT	1
P2X6Y6~	Australia - HR	H4	P2:90:1	TTG	-90	10	GAACT	1
P2X6Y8~	Australia - HR	H4	P2:90:1	TTG	-90	10	GAACT	1
P6X3Y10~	Australia - HR	H4	P2:90:1	TTG	-90	10	GAACT	1
S2X1Y3(2)~	Australia - HR	H4	P2:90:1	TTG	-90	10	GAACT	1
S2X2Y2~	Australia - HR	H4	P2:90:1	TTG	-90	10	GAACT	1
S2X6Y3~	Australia - HR	H4	P2:90:1	TTG	-90	10	GAACT	1
S3X5Y10(2)~	Australia - HR	H4	P2:90:1	TTG	-90	10	GAACT	1
S6X4Y8~	Australia - HR	H4	P2:90:1	TTG	-90	10	GAACT	1
17FRG089*	Australia - HR	H3	P2:46:5	TTA	-46	10	CTTTA	5
S6X2Y10	Australia - MR	H1	P1:77:4	TTC	-77	5	AAGTA	4
P2X2Y3	Australia - MR	H1	P1:75:1	TTC	-75	2.5	GTAGA	1
S6X5Y1	Australia - MR	H1	P1:75:1	TTC	-75	2.5	GTAGA	1
P2X4Y1	Australia - MR	H1	P1:74:3	TTC	-74	2.5	TAGAG	3
P2X5Y9(2)	Australia - MR	H1	P1:74:3	TTC	-74	2.5	TAGAG	3
P2X6Y7(2)	Australia - MR	H1	P1:74:3	TTC	-74	2.5	TAGAG	3
P3X2Y10	Australia - MR	H1	P1:74:3	TTC	-74	5	TAGAG	3
P3X6Y10(2)	Australia - MR	H1	P1:74:3	TTC	-74	2.5	TAGAG	3
S2X1Y7	Australia - MR	H1	P1:74:3	TTC	-74	2.5	TAGAG	3

S3X4Y9(2)	Australia - MR	H1	P1:74:3	TTC	-74	2.5	TAGAG	3
S6X1Y5	Australia - MR	H1	P1:74:3	TTC	-74	2.5	TAGAG	3
S6X2Y4	Australia - MR	H1	P1:74:3	TTC	-74	2.5	TAGAG	3
P2X1Y9	Australia - MR	H1	P1:66:3	TTC	-66	2.5	CATTT	3
18FRG195*	Australia - MR	H5	P1	CTC	-	2.5	-	
S2X2Y4	Australia - MR	H6	P1	CTC	-	2.5	-	
20P3003	Australia - S	H1	P1	TTC	-	0	-	
M3~	Australia - S	H2	P2	TTC	-	0	-	
P2X2Y9	Australia - S	H1	P1	TTC	-	0	-	
P2X3Y6	Australia - S	H1	P1	TTC	-	0	-	
P2X3Y8	Australia - S	H1	P1	TTC	-	0	-	
P2X4Y6	Australia - S	H1	P1	TTC	-	0	-	
P3X2Y9	Australia - S	H10	P2	TTC	-	0	-	
P3X6Y8	Australia - S	H1	P1	TTC	-	0	-	
S2X6Y8	Australia - S	H1	P1	TTC	-	0	-	
S3X3Y10	Australia - S	H1	P1	TTC	-	0	-	
S3X3Y7	Australia - S	H1	P1	TTC	-	0	-	
S6X1Y2	Australia - S	H1	P1	TTC	-	0	-	
S6X2Y5	Australia - S	H1	P1	TTC	-	0	-	
S6X3Y10	Australia - S	H1	P1	TTC	-	0	-	
S6X5Y4	Australia - S	H1	P1	TTC	-	0	-	
SG1	Australia - S	H1	P1	TTC	-	0	-	
H1016	Hungary - Untested	H12	P2:90:1	CTC	-46	NA	CTTTA	5
H801	Hungary - Untested	H7	P2:46:5	CTC	-46	NA	CTTTA	5
H917	Hungary - Untested	H12	P2:46:5	CTC	-46	NA	CTTTA	5
H918	Hungary - Untested	H12	P2:46:5	CTC	-46	NA	CTTTA	5
H933	Hungary - Untested	H12	P2:46:5	CTC	-46	NA	CTTTA	5
H947	Hungary - Untested	H12	P2:46:5	CTC	-46	NA	CTTTA	5
H953	Hungary - Untested	H12	P2:46:5	CTC	-46	NA	CTTTA	5
H812	Hungary - Untested	H14	P3	TTC	-			
CG16027	RSA - HR	H13	P2:95:2	CTC	-95	10	GCTAA	2

CG16033	RSA - HR	H13	P2:95:2	CTC	-95	10	GCTAA	2
CG16059	RSA - HR	H9	P2:95:2	CTC	-95	10	GCTAA	2
CG16060	RSA - HR	H9	P2:95:2	CTC	-95	10	GCTAA	2
CG16066	RSA - HR	H13	P2:95:2	CTC	-95	10	GCTAA	2
CG16045	RSA - HR	H4	P2:94:1	TTG	-94	10	CTAAG	1
CG16020	RSA - HR	H9	P2:90:1	CTC	-90	10	GAACT	1
CG16039	RSA - HR	H9	P2:90:1	CTC	-90	10	GAACT	1
CG16046	RSA - HR	H9	P2:90:1	CTC	-90	10	GAACT	1
CG16022	RSA - HR	H9	P2:74:2	CTC	-74	10	TAGAG	2
CG16069	RSA - HR	H9	P2:66:4	CTC	-66	10	AAGTG	4
CG16071	RSA - HR	H9	P2:66:4	CTC	-66	10	AAGTG	4
CG16025	RSA - MR	H10	P2:95:2	TTC	-95	2.5	GCTAA	2
CG16026	RSA - MR	H10	P2:95:2	TTC	-95	2.5	GCTAA	2
PTM21	RSA - Untested	H9	P2:95:2	CTC	-95	NA	GCTAA	2
PTM22	RSA - Untested	H9	P2:95:2	CTC	-95	NA	GCTAA	2
PTM23	RSA - Untested	H9	P2:95:2	CTC	-95	NA	GCTAA	2
PTM27#1	RSA - Untested	H9	P2:95:2	CTC	-95	NA	GCTAA	2
PTM56#2	RSA - Untested	H9	P2:95:2	CTC	-95	NA	GCTAA	2
PTM62#2	RSA - Untested	H9	P2:95:2	CTC	-95	NA	GCTAA	2
PTM67#2	RSA - Untested	H9	P2:95:2	CTC	-95	NA	GCTAA	2
PTM71	RSA - Untested	H9	P2:95:2	CTC	-95	NA	GCTAA	2
PTM73	RSA - Untested	H9	P2:95:2	CTC	-95	NA	GCTAA	2
PTM18	RSA - Untested	H13	P2:94:1	CTC	-94	NA	CTAAG	1
PTM14	RSA - Untested	H9	P2:90:1	CTC	-90	NA	GAACT	1
PTM24	RSA - Untested	H4	P2:90:1	TTG	-90	NA	GAACT	1
PTM32	RSA - Untested	H9	P2:90:1	CTC	-90	NA	GAACT	1
PTM55#2	RSA - Untested	H11	P2:90:4	TTA	-90	NA	GAACT	4
PTM57#1	RSA - Untested	H17	P2:90:1	TTG	-90	NA	GAACT	1
PTM58#2	RSA - Untested	H9	P2:90:1	CTC	-90	NA	GAACT	1
PTM64#1	RSA - Untested	H9	P2:90:1	CTC	-90	NA	GAACT	1
PTM69#2	RSA - Untested	H9	P2:90:1	CTC	-90	NA	GAACT	1

PTM15#1	RSA - Untested	H13	P2:66:3	CTC	-66	NA	AAGTG	3
PTM68#1	RSA - Untested	H9	P2:66:4	CTC	-66	NA	AAGTG	4
PTM25	RSA - Untested	H9	P2:62:4	CTC	-62	NA	TCATC	4
PTM26	RSA - Untested	H9	P2:62:4	CTC	-62	NA	TCATC	4
PTM38	RSA - Untested	H9	P2:62:4	CTC	-62	NA	TCATC	4
PTM19	RSA - Untested	H9	P2:57:3	CTC	-57	NA	CTATC	3
Gps 76	Turkey - S	H15	P5	TTC	-	0	-	
13-163	Turkey - S	H16	P4	TTC	-	0	-	
13-181	Turkey - S	H8	P3	TTC	-	0	-	
Gps 43	Turkey - S	H18	P6	TTC	-	0	-	

*Data and tebuconazole resistance phenotype inferred from Mair *et al*. 2020

~ RSA-like Australian isolates, as defined in STRUCTURE v 2.3.4 (Pritchard et al. 2000), with a RSA similarity Q-score of > 0.20 at K = 4.

Supplemental Table 6. *Ptm Cyp51A* haplotypes and their codon 489 nucleotide composition.

***Ptm Cyp51A* haplotype name**	**489 codon sequence**	**F489L non-synonymous mutation**	**Codon 489 notation in Knight *et al.*, 2023[1]**	**Source**	**GenBank ID**
H1	TTC	N	-	Mair *et al.*, 2020[2]	MT499776
H2	TTC	N	-	Mair *et al.*, 2020[2]	MT499777
H3	TTA	Y	L489-1	Mair *et al.*, 2020[2]	MT499778
H4	TTG	Y	L489-2	Mair *et al.*, 2020[2]	MT499779
H5	CTC	Y	L489-3	Mair *et al.*, 2020[2]	MT499780
H6	CTC	Y	L489-3	This study	OR734728
H7	CTC	Y	L489-3	This study	OR734729
H8	TTC	N	-	This study	OR734732
H9	CTC	Y	L489-3	This study	OR734726
H10	TTC	N	-	This study	OR734727
H11	TTA	Y	L489-1	This study	OR734725
H12	CTC	Y	L489-3	This study	OR734731
H13	CTC	Y	L489-3	This study	OR734730
H14	TTC	N	-	This study	OR734724
H15	TTC	N	-	This study	OR734722
H16	TTC	N	-	This study	OR734723
H17	TTG	Y	L489-2	This study	OR751399
H18	TTC	N	-	This study	OR751400

[1]Knight NL, Adhikari KC, Dodhia K, Mair WJ, Lopez-Ruiz FJ (2023) Workflows for detecting fungicide resistance in net form and spot form net blotch pathogens. bioRxiv:2023.2004.2027.538624

[2]Mair WJ, Thomas GJ, Dodhia K, Hills AL, Jayasena KW, Ellwood SR, Oliver RP, Lopez-Ruiz FJ (2020) Parallel evolution of multiple mechanisms for demethylase inhibitor fungicide resistance in the barley pathogen *Pyrenophora teres* f. sp. *maculata*. Fungal Genetics and Biology 145:1087-1845

Supplemental Table 7. *Ptm Cyp51A* promoter transposon insertion haplotypes. Haplotypes are named based on the two backbone DMI sensitive haplotypes (P1 and P2, Mair et al. 2020) from which haplotypes conferring resistance are derived, followed by the promoter transposon insertion position relative to the *Cyp51A* start codon, then the *Ty1-Copia* LTR retrotransposon-like insertion allele number. This convention distinguishes different transposon alleles at the same position.

Promoter haplotype name	Base haplotype	Transposon insertion position	Transposon allele	Notation in Mair *et al.*, 2020[1]	Notation in Knight *et al.*, 2023[2]	GenBank ID
P2:95:2	P2	-95	2			OR734247
P2:94:1	P2	-94	1			OR734245
P2:90:1	P2	-90	1	P7	PtTi-5	MT499787
P2:90:4	P2	-90	4			OR734246
P1:89:1	P1	-89	1		PtTi-8	OP753352
P1:77:4	P1	-77	4			OR734249
P1:75:1	P1	-75	1	P4	PtTi-3	MT499784
P2:74:2	P2	-74	2			OR734241
P2:74:3	P1	-74	3	P3	PtTi-1	MT499783
P1:69:4	P1	-69	4		PtTi-7	OP753351
P1:66:1	P1	-66	1		PtTi-6	OP753350
P1:66:3	P1	-66	3	P5	PtTi-4	MT499785
P2:66:3	P2	-66	3			OR734242
P2:66:4	P2	-66	4			OR734239
P2:62:4	P2	-62	4			OR734244
P2:57:3	P2	-57	3			OR734243
P2:46:5	P2	-46	5	P6	PtTi-2	MT499786

[1]Mair WJ, Thomas GJ, Dodhia K, Hills AL, Jayasena KW, Ellwood SR, Oliver RP, Lopez-Ruiz FJ (2020) Parallel evolution of multiple mechanisms for demethylase inhibitor fungicide resistance in the barley pathogen *Pyrenophora teres* f. sp. *maculata*. Fungal Genetics and Biology 145:1087-1845

[2]Knight NL, Adhikari KC, Dodhia K, Mair WJ, Lopez-Ruiz FJ (2023) Workflows for detecting fungicide resistance in net form and spot form net blotch pathogens. bioRxiv:2023.2004.2027.538624

Chapter 4 Supplementary files:

Supplemental Table 8. Metadata for *Pyrenophora* isolates genotyped in this study.

Isolate name	Host[a]	Locality[b]	Storage[c]	Year[d]	Symptom[f]	Latitude	Longitude
20BG2001	BG	PER	20BG2001	2020	Spot	-32.0071	115.87478
20BG2002	BG	PER	20BG2002	2020	Spot	-32.0071	115.87478
20BG2003	BG	PER	20BG2003	2020	Spot	-32.0071	115.87478
20BG2004	BG	PER	20BG2004	2020	Spot	-32.0071	115.87478
20BG2005	BG	PER	20BG2005	2020	Spot	-32.0071	115.87478
B-EMB-17A	B	EMB	17A	2021	Net	-34.376354	117.550138
B-EMB-17B	B	EMB	17B	2021	Spot	-34.376354	117.550138
B-EMB-17C	B	EMB	17C	2021	Spot	-34.376354	117.550138
B-EMB-19C	B	EMB	19C	2021	Spot	-34.516512	118.014694
B-EMB-19D	B	EMB	19D	2021	Spot	-34.516512	118.014694
B-EMB-19E	B	EMB	19E	2021	Spot	-34.516512	118.014694
B-EMB-24A	B	EMB	24A	2021	Spot	-34.516512	118.014695
B-EMB-24B	B	EMB	24B	2021	Spot	-34.516512	118.014695
B-EMB-25A	B	EMB	25A	2021	Net	-34.502977	117.868814
B-EMB-25E	B	EMB	25E	2021	Spot	-34.502977	117.868814
B-EMB-26A	B	EMB	26A	2021	Net	-34.502977	117.868814
B-EMB-26B	B	EMB	26B	2021	Net	-34.502977	117.868814
B-EMB-26C	B	EMB	26C	2021	Net	-34.502977	117.868814
B-EMB-26D	B	EMB	26D	2021	Net	-34.502977	117.868814
B-EMB-26F	B	EMB	26F	2021	Net	-34.502977	117.868814
B-EMB-26G	B	EMB	26G	2021	Net	-34.502977	117.868814
B-EMB-27A	B	EMB	27A	2021	Spot	-34.562977	117.868814
BG-BOS-4A	BG	BOS	4A	2021	Spot	-33.70307	117.11466
BG-BOS-4B	BG	BOS	4B	2021	Spot	-33.70307	117.11466
BG-BOS-4C	BG	BOS	4C	2021	Net	-33.70307	117.11466
BG-BOS-4D	BG	BOS	4D	2021	Net	-33.70307	117.11466
BG-BOS-4E	BG	BOS	4E	2021	Spot	-33.70307	117.11466
BG-BOS-4F	BG	BOS	4F	2021	Net	-33.70307	117.11466
BG-BOS-4G	BG	BOS	4G	2021	Net	-33.70307	117.11466
BG-BOS-4J	BG	BOS	4J	2021	Net	-33.70307	117.11466
BG-BOS-4K	BG	BOS	4K	2021	Spot	-33.70307	117.11466
BG-BOS-4L	BG	BOS	4L	2021	Net	-33.70307	117.11466
BG-EMB-10A	BG	EMB	10A	2021	Net	-34.57179	117.63608
BG-EMB-10B	BG	EMB	10B	2021	Net	-34.57179	117.63608
BG-EMB-10C	BG	EMB	10C	2021	Net	-34.57179	117.63608
BG-EMB-10D	BG	EMB	10D	2021	Net	-34.57179	117.63608
BG-EMB-10F	BG	EMB	10F	2021	Net	-34.57179	117.63608
BG-EMB-12A	BG	EMB	12A	2021	Spot	-34.50294	117.56422
BG-EMB-14A	BG	EMB	14A	2021	Net	-34.57179	117.63608
BG-EMB-14B	BG	EMB	14B	2021	Net	-34.57179	117.63608
BG-EMB-14D	BG	EMB	14D	2021	Net	-34.57179	117.63608
BG-EMB-14F	BG	EMB	14F	2021	Net	-34.57179	117.63608
BG-EMB-20A	BG	EMB	20A	2021	Net	-34.50298	117.86881
BG-EMB-20B	BG	EMB	20B	2021	Net	-34.50298	117.86881

BG-EMB-20D	BG	EMB	20D	2021	Spot	-34.50298	117.86881
BG-EMB-20E	BG	EMB	20E	2021	Spot	-34.50298	117.86881
BG-EMB-20F	BG	EMB	20F	2021	Spot	-34.50298	117.86881
BG-EMB-20G	BG	EMB	20G	2021	Spot	-34.50298	117.86881
BG-EMB-21A	BG	EMB	21A	2021	Spot	-34.50298	117.86881
BG-EMB-21B	BG	EMB	21B	2021	Spot	-34.50298	117.86881
BG-EMB-21C	BG	EMB	21C	2021	Spot	-34.50298	117.86881
BG-EMB-21D	BG	EMB	21D	2021	Spot	-34.50298	117.86881
BG-EMB-21F	BG	EMB	21F	2021	Spot	-34.50298	117.86881
BG-EMB-21G	BG	EMB	21G	2021	Spot	-34.50298	117.86881
BG-EMB-22A	BG	EMB	22A	2021	Spot	-34.50298	117.86881
BG-EMB-22B	BG	EMB	22B	2021	Spot	-34.50298	117.86881
BG-EMB-23A	BG	EMB	23A	2021	Spot	-34.50298	117.86881
BG-EMB-23C	BG	EMB	23C	2021	Spot	-34.50298	117.86881
BG-EMB-28B	BG	EMB	28B	2021	Net	-34.50298	117.86881
BG-EMB-28C	BG	EMB	28C	2021	Net	-34.50298	117.86881
BG-EMB-28F	BG	EMB	28F	2021	Net	-34.50298	117.86881
BG-EMB-28H	BG	EMB	28H	2021	Net	-34.50298	117.86881
BG-EMB-8A	BG	EMB	8A	2021	Net	-34.5048	117.88019
BG-EMB-8B	BG	EMB	8B	2021	Net	-34.5048	117.88019
BG-EMB-8C	BG	EMB	8C	2021	Net	-34.5048	117.88019
BG-EMB-8D	BG	EMB	8D	2021	Net	-34.5048	117.88019
BG-EMB-8E	BG	EMB	8E	2021	Net	-34.5048	117.88019
BG-EMB-8F	BG	EMB	8F	2021	Net	-34.5048	117.88019
BG-EMB-8G	BG	EMB	8G	2021	Net	-34.5048	117.88019
BG-EMB-8I	BG	EMB	8I	2021	Net	-34.5048	117.88019
BG-EMB-9A	BG	EMB	9A	2021	Net	-34.5032	117.58117
BG-EMB-9B	BG	EMB	9B	2021	Net	-34.5032	117.58117
BG-KOJ-11A	BG	KOJ	11A	2021	Net	-33.83061	117.16538
BG-KOJ-11B	BG	KOJ	11B	2021	Net	-33.83061	117.16538
BG-KOJ-11D	BG	KOJ	11D	2021	Net	-33.83838	117.161
BG-KOJ-13A	BG	KOJ	13A	2021	Spot	-33.83838	117.161
BG-KOJ-13B	BG	KOJ	13B	2021	Spot	-33.83838	117.161
BG-KOJ-13D	BG	KOJ	13D	2021	Spot	-33.83838	117.161
BG-KOJ-13E	BG	KOJ	13E	2021	Spot	-33.83838	117.161
BG-NBA-15A	BG	NBA	15A	2021	Net	-32.58049	116.44443
BG-NBA-15B	BG	NBA	15B	2021	Spot	-32.58049	116.44443
BG-NBA-15D	BG	NBA	15D	2021	Spot	-32.58049	116.44443
BG-NBA-15E	BG	NBA	15E	2021	Spot	-32.58049	116.44443
BG-NBA-15F	BG	NBA	15F	2021	Spot	-32.58049	116.44443
BG-NBA-15H	BG	NBA	15H	2021	Spot	-32.58049	116.44443
BG-NBO-5A	BG	NBO	5A	2021	Net	-33.52233	117.07139
BG-NKO-7A	BG	NKO	7A	2021	Spot	-33.77326	117.14047
BG-NKO-7B	BG	NKO	7B	2021	Spot	-33.77326	117.14047
BG-NKO-7C	BG	NKO	7C	2021	Spot	-33.77326	117.14047
BG-PER-30B	BG	PER	30B	2021	Net	-32.0071	115.87478
BG-PER-30C	BG	PER	30C	2021	Net	-32.0071	115.87478
BG-SBO-1A	BG	SBO	1A	2021	Net	-33.72276	117.10568

BG-SBO-1B	BG	SBO	1B	2021	Net	-33.72276	117.10568
BG-SBO-1C	BG	SBO	1C	2021	Net	-33.72276	117.10568
BG-SBO-1D	BG	SBO	1D	2021	Net	-33.72276	117.10568
BG-SBO-1E	BG	SBO	1E	2021	Net	-33.72276	117.10568
BG-SBO-1G	BG	SBO	1G	2021	Net	-33.72276	117.10568
BG-SBO-1I	BG	SBO	1I	2021	Net	-33.72276	117.10568
BG-SBO-2B	BG	SBO	2B	2021	Net	-33.52233	117.07139
BG-SBO-2C	BG	SBO	2C	2021	Net	-33.52233	117.07139
BG-SBO-2D	BG	SBO	2D	2021	Net	-33.52233	117.07139
BG-SBO-2G	BG	SBO	2G	2021	Net	-33.52233	117.07139
B-KOJ-16A	B	KOJ	16A	2021	Net	-33.70307	117.11466
B-KOJ-16B	B	KOJ	16B	2021	Net	-33.70307	117.11466
B-KOJ-16C	B	KOJ	16C	2021	Net	-33.70307	117.11466
B-KOJ-16D	B	KOJ	16D	2021	Spot	-33.70307	117.11466
B-KOJ-16E	B	KOJ	16E	2021	Net	-33.70307	117.11466
B-KOJ-18A	B	KOJ	18A	2021	Net	-33.722763	117.108108
B-KOJ-18B	B	KOJ	18B	2021	Spot	-33.722763	117.108108
B-KOJ-18C	B	KOJ	18C	2021	Spot	-33.722763	117.108108
B-KOJ-18D	B	KOJ	18D	2021	Spot	-33.722763	117.108108
B-KOJ-18E	B	KOJ	18E	2021	Spot	-33.722763	117.108108
B-KOJ-18F	B	KOJ	18F	2021	Spot	-33.722763	117.108108
B-KOJ-18G	B	KOJ	18G	2021	Net	-33.722763	117.108108
B-KOJ-18H	B	KOJ	18H	2021	Net	-33.722763	117.108108
NB29	B	-	NB29	1985	Net*	-30.830454	116.7009099
SG1	B	-	SG1	1996	Spot*	-30.444044	115.614800
W11	B	-	Won 1-1	2009	Net*	-30.444044	115.614800
17-058	B	-	ptm17-058	2017	Spot	-35.997320	142.925496

[a] Original host of the isolate B: Barley (*Hordeum vulgare*), BG: Barley grass (*Hordeum murinum* L).
[b] General region assignment isolate locations in study.
[c] Alternate isolate name in storage.
[e] Isolate collection year.
[f] Symptomatic form on host, determined at time of collection * Type isolates unknown method of determination.

Supplemental Table 9. PhiPT values between isolate hosts and forms/ types.

	Barley grass Net	Barley grass Spot	Barley Spot	Barley Net
Barley grass net	0.000			
Barley grass Spot	0.029	0.000		
Barley Spot	0.933	0.916	0.000	
Barley Net	0.928	0.911	0.872	0.000

Genetic distances between isolate host and forms.

Concise R code used in analysis.

```
#Required R libraries to run most analyses.
library("poppr")
library("magrittr")
library("DartR")
library("adegenet")
library("ggplot2")
library("dplyr")
library("ade4")

#Additional packages required to run dendrogram to map figures.
library("mapdata")
library("maps")
library("devtools")
library("phytools")
library("ozmaps")
library("sf")


#Import data and filter.
#Get Genalex marker file
x = getfile()
x = read.genalex(x$files, ploidy = 1)
#Remove loci with missing data >5%, genotypes with missing data >10%, retain only informative loci.
x = x %>% missingno("loci", cutoff = 0.05)
x = x %>% missingno("geno", cutoff = 0.1)
x = informloci(x)
#Determine genetic distance between isolates and between biological/ technical replicates.
myDist <- provesti.dist(x)
myDist
#Filter isolates by determined genetic distance, to assign multilocus genotypes (MLGs). Value assigned in example = 0.025.
mlg.filter(x, threshold = 0.025, distance = myDist, threads = 1L)
mlg.filter(x, distance = myDist) <- 0.025
mlg.vector(x, reset = FALSE)
mlg.filter(x, threshold = 0.025, distance = myDist, threads = 1L)
x
#View table of MLGs.
x.tab <- mlg.table(x, total = T, background = T)
x.tab
#Clone correct population for Principal components analysis (PCA) and phylogenetic tree.
x.cc <- clonecorrect(x, strata =~Pop)
x.cc
#Output filtered data.
genind2genalex(x.cc)

#Generate population genetic diversity indices.
x_diversity = poppr(x)
x_diversity

#Generate phylogenetic tree with 1000 iterations.
```

```
tree = aboot(x, dist = provesti.dist, sample = 1000, cutoff = 0, quiet = TRUE)

#Generate alternative file types for different packages. First generate Genind object, then Genlight.
x_GI = genclone2genind(x)
x_GL = gi2gl(x_GI)

#Generate minimum spanning networks.
x.msn <- poppr.msn(x_GI, AnkeSNP.dist, showplot = T, include.ties = T, vertex.label = NA)

#Generate minimum spanning networks with GUI.
imsn()

#Conduct PCA.
#Generate bar plot of each PC.
x.pca <- glPca(x_GL, nf = 10)
barplot(100*x.pca$eig/sum(x.pca$eig), col = heat.colors(170), main="PCA Eigenvalues")
title(ylab="Percent of variance explained", line = 2)
title(xlab="Eigenvalues", line = 1)
#Determine each PC to 4 decimal significant figures. y = the PC you wish to determine.
var_frac <- x.pca$eig/sum(x.pca$eig)
signif(sum(var_frac[y])*100, 4)
#Generate scores for PCA plot.
x.pca.scores <- as.data.frame(x.pca$scores)
x.pca.scores$pop <- pop(x_GL)
x.pca.scores
#Generate PCA figure.
p  <- ggplot(x.pca.scores, aes(x=PC1, y=PC2, color = pop))
p <- p + geom_point(size=5)
p <- p + stat_ellipse(level = 0.95, size = 1)
p <- p + scale_color_manual(values = colz)
p <- p + geom_hline(yintercept = 0)
p <- p + geom_vline(xintercept = 0)
p <- p + theme_bw()
p <- p + geom_label_repel(aes(label = rownames(x.pca.scores), size = NULL), nudge_y = 1)
p

#Cluster analysis.
#Find appropriate number of clusters to retain.
grp <- find.clusters(x, max.n.clust=8)
#Perform and plot discriminant analysis of principal components DAPC.
newdapc =  dapc(x, grp$grp)
scatter(newdapc, scree.pca = F, scree.da = T, legend = T, solid = 1)
#Determine markers which contribute most to population structure.
contrib <- loadingplot(newdapc$var.contr, axis = 1, thres = 0.07, lab.jitter = 1)
#Determine appropriate PC to retain in DAPC.
system.time(pramx <- xvalDapc(tab(x, NA.method = "mean"), pop(x),
                      n.pca = 1:70, n.rep = 10,
                      parallel = "snow", ncpus = 4L))

#AMOVA tests.
#Split populations into subpopulation for analysis, different types of analysis listed.
```

```
splitStrata(x) <- ~Region/Year
splitStrata(x) <- ~Symptom/Location
splitStrata(x) = ~Variety/Agzone/Field
x.amova = poppr.amova(x, ~Host/Symptom)

#Link dendrogram to maps for figures.
#Obtain world data. Obtain isolate location data.
data(world)
loc <- read.csv("C:/Users…", sep=",", row.names = 1)
#Map isolate locations, to regions with phylogenetic tree generated previously.
phymap <- phylo.to.map(tree, loc, database="worldHires", regions="AU", direction="leftwards", ftype="i")

#Generate ggplot2 based maps in chapter 1.
#obtain map in form of shape file.
shp <- read_sf(dsn = file.path("C:/Users… .shp"), stringsAsFactors = F)
plot(shp)
shp <- fortify(shp)
#Use ggplot to map co-ordinates. Metadata file includes columns, for lat and long co-ordinates and column for field.
metadata <- read.table("C:/Users… .csv", sep=",", header=T)
main_map = ggplot(shp) + geom_sf(aes(fill = agzone), color = NA) +
geom_point(data = metadata,  mapping = aes(x = lon, y = lat, color = factor(field)), size = 3) +
scale_fill_manual(values = c("colours for each agzone")) +
labs(fill="Agzone")+
labs(title = "WA Isolate Locations", subtitle = "With agricultural regions.") +
labs(color='Study') +
xlab("Latitude") +
ylab("Longitude") +
coord_sf()+
theme_bw()
main_map
```

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